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(54) Title: CC CHEMOKINE RECEPTOR 5, ANTIBODIES THERETO, TRANSGENIC ANIMALS

(57) Abstract

The susceptibility of human macrophages to human immunodeficiency virus (HIV) infection depends on cell surface expression of the human CD4 molecule and CC cytokine receptor 5. CCR5 is a member of the 7-transmembrane segment superfamily of G-protein-coupled cell surface molecules. CCR5 plays an essential role in the membrane fusion step of infection by some HIV isolates. The establishment of stable, nonhuman cell lines and transgenic mammals having cells that coexpress human CD4 and CCR5 provides valuable tools for the continuing research of HIV infection. In addition, antibodies which bind to CCR5, CCR5 variants, and CCR5-binding agents, capable of blocking membrane fusion between HIV and target cells represent potential anti-HIV therapeutics for macrophage-tropic strains of HIV.

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Exhibit 1

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CC CHEMOKINE RECEPTOR 5, ANTIBODIES THERETO, TRANSGENIC ANIMALS**INFECTION****FIELD OF THE INVENTION**

5 The present invention relates to *in vitro* and *in vivo* models for the study of human immunodeficiency virus (HIV) infection and the effectiveness of anti-HIV therapeutics. The invention more specifically relates to cell surface proteins that participate in HIV infection and which are useful for the development of animal models.

BACKGROUND OF THE INVENTION

10 An HIV infection cycle begins with the entry of an HIV virus into a target cell. Entry commences when an HIV envelope glycoprotein (*env*) binds to a human CD4 molecule in a target cell membrane. This binding leads to fusion of virus and cell membranes, which in turn facilitates virus entry into the host. The HIV-infected host cell eventually expresses *env* on its surface. This expression allows the infected cell to fuse with uninfected, CD4-positive cells, thereby spreading the virus.

15 Recent studies have shown that the HIV fusion process occurs with a wide range of human cell types that either express human CD4 endogenously or that have been engineered to express human CD4. The fusion process, however, does not occur with nonhuman cell types engineered to express human CD4 even though these nonhuman cells still can bind *env*.
20 The disparity between human and nonhuman cell types exists because membrane fusion requires the coexpression of human CD4 and one or more cofactors specific to human cell types. Nonhuman cell types that have been engineered to express human CD4 but not the additionally required factor(s) are incapable of membrane fusion, and therefore are nonpermissive for HIV infection.

25 Some individual HIV isolates, designated "macrophage-tropic," efficiently infect primary macrophages but not immortalized T-cell lines. Other isolates, designated "T-cell

line-tropic," have the opposite property and infect immortalized T-cell lines more efficiently than they infect primary macrophages. Both types of isolates readily infect primary T-cells from the body, however. The selective tropism of these two types of isolates is thought to be due to their requirements for distinct cofactors that are differentially expressed on different CD4 positive cell types. It should be understood that other HIV strains are "dual-tropic" and have the ability to infect both macrophages and immortalized T-cell lines and are believed to be able to use more than one cofactor.

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Recently a cofactor required for fusion of virus and cell membranes has been described. Feng *et al.*, *Science* 272: 872-7 (1996). This factor, called "fusin," (also known as CXCR4) permits cells that contain human CD4 to fuse with the surface of an HIV virus. Fusin functions preferentially for T-cell line-tropic HIV-1 isolates and much less well for macrophage-tropic HIV-1 isolates.

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The discovery of fusin allows the creation of a successful small animal model. Such a model is crucial for studies of HIV infection and of the effectiveness of anti-HIV therapeutics. But the presence of fusin enables the study of T-cell line-tropic but not macrophage-tropic isolates. This is an important distinction because macrophage-tropic isolates represent the predominant type of isolates obtained from infected individuals. Macrophage-tropic isolates also appear to be preferentially transmitted between individuals. A putative cofactor that is necessarily expressed with CD4 to allow entry of macrophage-tropic isolates remains unknown.

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In recent years, researchers have bred transgenic animals that contain cells which express human CD4 and which could be used as models for HIV infection of macrophages if the macrophage-specific factor were known. See, for example, Dunn *et al.*, *Human immunodeficiency virus type 1 infection of human CD4-transgenic rabbits*, J. Gen. Vir. 76:1327-1336 (1995); Snyder *et al.*, *Development and Tissue-Specific Expression of Human CD4 in Transgenic Rabbits*, Mol. Reprod. & Devel. 40:419-428 (1995); Killeen *et al.*, *Regulated Expression of Human CD4 Rescues Helper T-Cell Development in Mice Lacking Expression of Endogenous CD4*, EMBO J. 12:1547-1553 (1993); Forte *et al.*, *Human CD4 Produced in Lymphoid Cells of Transgenic Mice Binds HIV gp120 and Modifies the Subsets of Mouse T-Cell Populations*, Immunogenetics 38:455-459 (1993).

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A goal of research in this field is to find a putative factor for the macrophage-tropic isolates that could be co-expressed with CD4 in a small animal. Such co-expression would provide an animal model to develop efficacious therapies to combat infection by macrophage-tropic HIV isolates. The discovery of other essential cofactors would provide new targets for development of anti-HIV therapies.

SUMMARY OF THE INVENTION

The present invention is based on the discovery of a new CC chemokine receptor protein associated with HIV infection (formerly referred to as "CC CKR5", now more commonly known as "CCR5"). The invention provides isolated polynucleotides and polypeptides encoded by CCR5 polynucleotides, as well as antibodies directed against regions of CCR5 and peptide fragments of CCR5 which block HIV interaction with the CC CKR5 receptor.

It is an object of the present invention to provide therapeutic and preventative medicinal agents effective against HIV infection and effective in regulating monocyte accumulation and activation. In accomplishing these and other objects, there has been provided, in accordance with one aspect of the present invention a stable, nonhuman cell line, the cells of which contain DNA encoding CCR5. In accordance with another aspect of the invention a transgenic non-human mammal is provided comprised of cells that coexpress human CD4 and CCR5.

In another aspect of the invention, the invention provides an antibody which binds to CCR5 and which blocks membrane fusion between HIV and a target cell. In accordance with another aspect of the invention, there is provided a cell that expresses a CCR5 gene, wherein the CCR5 gene is not stably integrated into the genome of said cell.

In accordance with yet another aspect of the invention an isolated and purified peptide fragment of CCR5 is provided that blocks membrane fusion between HIV and a target cell.

In yet another aspect, the invention provides a method for identifying a compound which blocks membrane fusion between HIV and a CCR5 target cell or between an HIV-infected cell and a CCR5 positive uninfected cell. The method includes the steps of : a) incubating components comprising the compound and a CDU and CCR5 positive cell under conditions sufficient to allow the components to interact; b) contacting the components of step a) with HIV or an HIV-infected cell; and c) measuring the ability of the compound to block membrane fusion between HIV and the CCR5 positive cell or between an HIV-infected cell and a CCR5 positive uninfected cell.

In accordance with yet another aspect of the invention a method of inhibiting CCR5 expression in a cell is provided, comprising introducing into the cell at least one antisense polynucleotide that causes the inhibition of CCR5 in the cell.

5 In accordance with yet another aspect of the invention is provided a CCR5-binding agent, wherein said agent blocks binding of a chemokine and HIV to CCR5.

The antibodies and blocking agents of the invention are also useful for providing methods for modulating an immune response in which macrophages are involved. For example, administration of CCR5 agonists or antagonists would be useful for modulating the immune response.

10 Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art 15 from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A shows an alignment of amino acid sequences deduced from cDNAs for CC CKR1, CC CKR2B, and for CCR5. Arabic numbers enumerate a CCR5 amino acid sequence (SEQ ID NO:4) and a variant with residue changed from alanine to leucine (SEQ ID NO: 2) that has been deduced from a CCR5 DNA sequence (SEQ ID NO:3 and SEQ ID NO: 1, respectively) and are left-justified. Putative membrane-spanning segments I-VII are noted. Vertical bars show identities between adjacent residues and open boxes show predicted sites for N-linked glycosylation. Dashes and gaps have been inserted to optimize the alignments. Extracellular portions of the CCR5 polypeptide are located between transmembrane domains 2 and 3, transmembrane domains 4 and 5, transmembrane domains 6 and 7, and in the amino terminal segment before transmembrane domain 1.

FIGURE 1B shows the nucleotide and deduced amino acid sequence (SEQ ID NO:1 and 2, respectively) for a CCR5 variant where nucleotides 293-296 of the wild-type DNA is changed from CTTG to TGCT resulting in a change at amino acid residue 127, from Alanine to Leucine.

FIGURE 1C shows the nucleotide and deduced amino acid sequence (SEQ ID NO:3 and 4, respectively) for CCR5.

FIGURE 2 shows CCR5 peptides which inhibit fusion between cells expressing the HIV-1 Env from the macrophage-tropic Ba-L isolate and murine cells co-expressing CD4 and CCR5. Peptides were preincubated with HIV-Env-expressing cells for 1 hour at a concentration from 0-50 µg/ml before mixing with cells which express CD4 and CCR5.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention originated from studies on receptor proteins of chemokines. The inventors cloned, sequenced, and functionally expressed a human cDNA encoding a novel macrophage-selective CC chemokine receptor that has been designated CCR5.

During their investigation, the inventors discovered that CCR5 is a necessary cofactor for infection by macrophage-tropic HIV isolates. More particularly, the inventors found that when they transgenically expressed human CCR5 in non-human cells which also transgenically express human CD4, the altered cells could fuse with cells that express the *env* envelope protein from macrophage-tropic strains of HIV. It should be understood that other HIV strains are "dual-tropic" and have the ability to infect both macrophages and immortalized T-cell lines and are believed to be able to use more than one cofactor. Furthermore, the inventors reasoned that antibodies against CCR5 can inhibit the fusion of cells that contain CD4 and CCR5, upon contact with cells that express the *env* protein from macrophage-tropic strains of HIV. Antibodies which bind CCR5 can inhibit infection of cells that contain CCR5 and CD4 by macrophage-tropic strains of HIV. The insights of the present invention enable the development of new tools to study HIV infection of macrophages and the discovery of new HIV treatment methodologies based on chemokine receptor biochemistry.

Chemokine receptors are thought to have seven transmembrane-domains, are coupled to G-protein and participate in cellular responses to chemokines. Receptor CCR5 that has been cloned by the inventors is the fifth human CC chemokine receptor identified to date. The five receptors bind overlapping but distinct subsets of CC chemokines. Of the five, only CC chemokine receptor 5 ("CCR5") displays a CC chemokine specificity profile that matches the profile for suppression of HIV-1 infection. Cocchi *et al.*, *Science* 270, 1811 (1995). RANTES, MIP-1 α and MIP-1 β are potent agonists of CCR5, but MCP-1 and MCP-3 are not, as summarized by Combadiere *et al.* in *J. Biol. Chem.* 270: 16491-4 (1995), *J. Biol. Chem.* 270: 30235 (1995), and *Molec. Biol. Cell.* 6: 224a (1995) and by Samson *et al.* in *Biochemistry* 35: 3362 (1996) the disclosures of which are incorporated herein in their entireties.

ISOLATION OF cDNA ENCODING CCR5

The gene for the chemokine receptor of the present invention can be cloned from a human cDNA library. Methods used to clone novel chemokine receptor-like cDNAs from a λgt11 cDNA library made from peripheral blood mononuclear cells of a patient with eosinophilic leukemia have been described by Combadiere *et al.*, *DNA Cell Biol.* 14: 673-80 (1995), which is herein incorporated in its entirety by reference. A cDNA encoding CCR5 also can be isolated by the procedure described by U.S. provisional patent application 60/010,854 filed on January 30, 1996, which is herein incorporated by reference.

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The above-described methods can be used to identify DNA sequences that code for one or more CCR5 polypeptide sequences. A nucleotide sequence determined by the inventors, herein described as SEQ ID NO:3 of the present invention, has been deposited with the Genbank/EMBL data libraries under accession number US7840. But many other related sequences that code for CCR5 and altered forms of CCR5 are contemplated in context of the various embodiments enumerated herein (e.g., SEQ ID NO:1).

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In preferred embodiments fusion between *env*-expressing effector cells and CD4-expressing and CCR5-expressing target cells, prepared by infection with vaccinia virus, induces activation of *Escherichia coli lacZ*, causing β-galactosidase production in fused cells as described by Nussbaum *et al.*, *J. Virol.* 68: 5411 (1994), which is incorporated in its entirety by reference. The specificity of cell fusion as measured with this assay is equivalent to the specificity of infection by HIV-1 virions.

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The invention provides an isolated polynucleotide sequence encoding a polypeptide having an amino acid sequence as set forth in SEQ ID NO:4. The term "isolated" as used herein includes polynucleotides substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which it is naturally associated. Polynucleotide sequences of the invention include DNA, cDNA and RNA sequences which encode CCR5. It is understood that all polynucleotides encoding all or a portion of CCR5 are also included herein, as long as they encode a polypeptide with CCR5 activity (e.g., act as a cofactor for HIV infection). Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, portions of the mRNA sequence may be altered due to alternate RNA splicing patterns or the use of alternate promoters for RNA transcription.

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As another example, CCR5 polynucleotide may be subjected to site-directed mutagenesis. The polynucleotide sequence for CCR5 also includes antisense sequences. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of CCR5 polypeptide encoded by the nucleotide sequence is functionally unchanged. Also included are nucleotide sequences which encode CCR5 polypeptide, such as SEQ ID NO:1. In addition, the invention also includes a polynucleotide encoding a polypeptide having the biological activity of an amino acid sequence of SEQ ID NO:4 and having at least one epitope for an antibody immunoreactive with CCR5 polypeptide. Assays provided herein which show association between HIV infection and expression of CCR5 can be used to detect CCR5 activity.

The polynucleotide encoding CCR5 includes the nucleotide sequence in FIGURE 1 (SEQ ID NO:1 and 3), as well as nucleic acid sequences complementary to that sequence. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxyribonucleotides A, G, C, and T of FIGURE 1 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments (portions) of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes the protein of FIGURE 1 (e.g., SEQ ID NO: 4). "Selective hybridization" as used herein refers to hybridization under moderately stringent or highly stringent physiological conditions (See, for example, the techniques described in Maniatis et al., 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference), which distinguishes related from unrelated nucleotide sequences.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, e.g., high stringency conditions, or each of the conditions can be used, e.g., for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

Specifically disclosed herein is a cDNA sequence for CCR5. SEQ ID NO:3 represents the wild-type sequence and SEQ ID NO:1 represents a cDNA which encodes CCR5 having a conservative substitution of Leucine for Alanine at amino acid residue 127. The result of this conservative variation should not affect biological activity of CCR5 polypeptide or peptides containing the variation (see Example 5).

DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization or computer-based techniques which are well known in the art. These include, but are not limited to: 1) hybridization of genomic or cDNA libraries with probes to detect homologous nucleotide sequences; 2) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features; 3) polymerase chain reaction (PCR) on genomic DNA or cDNA using primers capable of annealing to the DNA sequence of interest; 4) computer searches of sequence databases for similar sequences; and 5) differential screening of a subtracted DNA library.

Preferably the CCR5 polynucleotide of the invention is derived from a mammalian organism. Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate.

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This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al.*, *Nucl. Acid Res.*, 9:879, 1981). Alternatively, a subtractive library, as illustrated herein is useful for elimination of non-specific cDNA clones.

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When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the synthesis of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al.*, *Nucl. Acid Res.*, 11:2325, 1983).

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A cDNA expression library, such as lambda gt11, can be screened indirectly for CCR5 peptides having at least one epitope, using antibodies specific for CCR5. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of CCR5 cDNA.

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Alterations in CCR5 nucleic acid include intragenic mutations (*e.g.*, point mutation, nonsense (stop), missense, splice site and frameshift) and heterozygous or homozygous deletions. Detection of such alterations can be done by standard methods known to those of skill in the art including sequence analysis, Southern blot analysis, PCR based analyses (*e.g.*,

multiplex PCR, sequence tagged sites (STSSs)) and *in situ* hybridization. Such proteins can be analyzed by standard SDS-PAGE and/or immunoprecipitation analysis and/or Western blot analysis, for example.

DNA sequences encoding CCR5 can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the CCR5 polynucleotide sequences may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the CCR5 genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, *et al.*, *Gene*, 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, *J. Biol. Chem.*, 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (*e.g.*, T7, metallothionein 1, or polyhedrin promoters).

Polynucleotide sequences encoding CCR5 can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. However, since mature CCR5 is glycosylated, the choice of host cells depends on whether or not the glycosylated or non-glycosylated form of CCR5 is desired. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication

in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the CCR5 coding sequence and appropriate transcriptional-/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques. (See, for example, the techniques described in Maniatis et al., 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.)

A variety of host-expression vector systems may be utilized to express the CCR5 coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the CCR5 coding sequence; yeast transformed with recombinant yeast expression vectors containing the CCR5 coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the CCR5 coding sequence; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the CCR5 coding sequence; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, retroviruses, adenovirus, vaccinia virus) containing the CCR5 coding sequence, or transformed animal cell systems engineered for stable expression. Since CCR5 has not been confirmed to contain carbohydrates, both bacterial expression systems as well as those that provide for translational and post-translational modifications may be used; *e.g.*, mammalian, insect, yeast or plant expression systems.

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see *e.g.*, Bitter et al., 1987, Methods in Enzymology 153:516-544). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage γ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or

from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted CCR5 coding sequence.

5 In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, *in* Methods in Enzymology, Eds. Wu & Grossman, 31987, Acad. Press, N.Y., Vol. 153, pp.516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II. A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used (Cloning in Yeast, Ch. 3, R. Rothstein *In:* DNA Cloning Vol.11, A Practical Approach, Ed. DM Glover, 1986, IRL Press, Wash., D.C.). Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

20 Eukaryotic systems, and preferably mammalian expression systems, allow for proper post-translational modifications of expressed mammalian proteins to occur. Eukaryotic cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, phosphorylation, and advantageously, plasma membrane insertion of the gene product may be used as host cells for the expression of CCR5.

25 Mammalian cell systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the CCR5 coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K promoter may be used. (*e.g.*, see, Mackett et al., 1982, Proc. Natl. Acad. Sci. USA 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. USA 79: 4927-4931). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, et al., 1981,

Mol. Cell. Biol. 1: 486). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as, for example, the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the CCR5 gene in host cells (Cone & Mulligan, 1984, *Proc. Natl. Acad. Sci. USA* 81:6349-6353). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothioneine II A promoter and heat shock promoters.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the CCR5 cDNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, *et al.*, 1977, *Cell* 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48: 2026), and adenine phosphoribosyltransferase (Lowy, *et al.*, 1980, *Cell* 22: 817) genes can be employed in tk-, hgprt or aprt cells respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, *et al.*, 1980, *Natl. Acad. Sci. USA* 77: 3567; O'Hare, *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78: 2072; neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, 1981, *J. Mol. Biol.* 150: 1); and hygro, which confers resistance to hygromycin (Santerre, *et al.*, 1984, *Gene* 30: 147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD,

which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, *Proc. Natl. Acad. Sci. USA* 85: 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: *Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory ed.).

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the CCR5 of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (see for example, *Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

15 *Cell Lines*

In one embodiment, the present invention relates to stable recombinant cell lines, the cells of which express CCR5 polypeptide or coexpress human CD4 and CCR5 and contain DNA that encodes CCR5. Suitable cell types include but are not limited to cells of the following types: NIH 3T3 (Murine), Mv 1 lu (Mink), BS-C-1 (African Green Monkey) and human embryonic kidney (HEK) 293 cells. Such cells are described, for example, in the Cell Line Catalog of the American Type Culture Collection (ATCC). These cells can be stably transformed by a method known to the skilled artisan. See, for example, Ausubel *et al.*, *Introduction of DNA Into Mammalian Cells*, in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, sections 9.5.1-9.5.6 (John Wiley & Sons, Inc. 1995). "Stable" transformation in the context of the invention means that the cells are immortal to the extent of having gone through at least 50 divisions.

CCR5 can be expressed using inducible or constitutive regulatory elements for such expression. Commonly used constitutive or inducible promoters, for example, are known in

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the art. The desired protein encoding sequence and an operably linked promoter may be introduced into a recipient cell either as a non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the desired molecule may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome. Therefore the cells can be transformed stably or transiently.

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An example of a vector that may be employed is one which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may complement an auxotrophy in the host (such as leu2, or ura3, which are common yeast auxotrophic markers), biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

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In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

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For a mammalian host, several possible vector systems are available for expression. One class of vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors include vaccinia virus expression vectors. A third class of vectors relies upon the integration of the desired gene sequences into the host chromosome. Cells which have stably integrated the introduced DNA into their chromosomes may be selected by also introducing one or more markers (e.g., an

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exogenous gene) which allow selection of host cells which contain the expression vector. The marker may provide for prototropy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper or the like. The selectable marker gene can either be directly linked to the DNA sequences to be expressed, or introduced into the same cell by co-transformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, H., Mol. Cell. Biol., 3:280 (1983), and others.

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Once the vector or DNA sequence containing the construct has been prepared for expression, the DNA construct may be introduced (transformed) into an appropriate host. Various techniques may be employed, such as protoplast fusion, calcium phosphate precipitation, electroporation or other conventional techniques.

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TRANSGENIC ANIMALS

In another embodiment, the present invention relates to transgenic animals having cells that coexpress human CD4 and CCR5. Such transgenic animals represent a model system for the study of HIV infection and the development of more effective anti-HIV therapeutics.

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The term "animal" here denotes all mammalian species except human. It also includes an individual animal in all stages of development, including embryonic and fetal stages. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice), and domestic pets (for example, cats and dogs) are included within the scope of the present invention.

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A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus. "Transgenic" in the present context does not encompass classical crossbreeding or *in vitro* fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the present invention also contemplates the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes.

The term "transgenic animal" also includes a "germ cell line" transgenic animal. A germ cell line transgenic animal is a transgenic animal in which the genetic information has been taken up and incorporated into a germ line cell, therefore conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they, too, are transgenic animals.

It is highly preferred that the transgenic animals of the present invention be produced by introducing into single cell embryos DNA encoding CCR5 and DNA encoding human CD4, in a manner such that these polynucleotides are stably integrated into the DNA of germ line cells of the mature animal and inherited in normal mendelian fashion. Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In a preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo.

In a most preferred method the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. These techniques are well known. For instance, reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian (mouse, pig, rabbit, sheep, goat, cow) fertilized ova include: Hogan *et al.*, MANIPULATING THE MOUSE EMBRYO (Cold Spring Harbor Press 1986); Krimpenfort *et al.*, *Bio/Technology* 9:86 (1991); Palmiter *et al.*, *Cell* 41:343 (1985); Kraemer *et al.*, GENETIC MANIPULATION OF THE EARLY MAMMALIAN EMBRYO (Cold Spring Harbor Laboratory Press 1985); Hammer *et al.*, *Nature*, 315:680 (1985); Purcel *et al.*, *Science*, 244:1281 (1986); Wagner *et al.*, U.S. patent No. 5,175,385; Krimpenfort *et al.*, U.S. patent No. 5,175,384, the respective contents of which are incorporated by reference.

The cDNA that encodes CCR5 can be fused in proper reading frame under the transcriptional and translational control of a vector to produce a genetic construct that is then amplified, for example, by preparation in a bacterial vector, according to conventional

methods. See, for example, the standard work: Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Press 1989), the contents of which are incorporated by reference. The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals.

5 Production of transgenic animals containing the gene for human CD4 have been described. See Snyder *et al.*, *supra*; Dunn *et al.*, *supra*, the contents of which are incorporated by reference.

10 The term "transgenic" as used herein additionally includes any organism whose genome has been altered by *in vitro* manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. The term "gene knockout" as used herein, refers to the targeted disruption of a gene *in vivo* with complete loss of function that has been achieved by any transgenic technology familiar to those in the art. In one embodiment, transgenic animals having gene knockouts are those in which the target gene has been rendered nonfunctional by an insertion targeted to the gene to be rendered non-functional by homologous recombination. As used herein, the term "transgenic" includes any transgenic technology familiar to those in the art which can produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional or "knocked out."

20 The transgene to be used in the practice of the subject invention is a DNA sequence comprising a modified CCR5 coding sequence. In a preferred embodiment, the CCR5 gene is disrupted by homologous targeting in embryonic stem cells. For example, the entire mature C-terminal region of the CCR5 gene may be deleted as described in the examples below. Optionally, the CCR5 disruption or deletion may be accompanied by insertion of or replacement with other DNA sequences, such as a non-functional CCR5 sequence. In other embodiments, the transgene comprises DNA antisense to the coding sequence for CCR5. In another embodiment, the transgene comprises DNA encoding an antibody or receptor peptide sequence which is able to bind to CCR5. Where appropriate, DNA sequences that encode proteins having CCR5 activity but differ in nucleic acid sequence due to the degeneracy of the genetic code may also be used herein, as may truncated forms, allelic variants and interspecies homologues.

ANTIBODIES WHICH BIND TO CCR5 INHIBIT FUSION

In another embodiment, the present invention relates to antibodies that bind CCR5 that block *env*-mediated membrane fusion (i) associated with HIV entry into a human CD4-positive target cell or (ii) between an HIV-infected cell and an uninfected human CD4-positive target cell. The invention also includes antibodies that bind to CCR5 and inhibit chemokine binding. For example, such antibodies may be useful for ameliorating immune response disorders associated with macrophages. Antibodies of the invention may also inhibit gp120 binding to CCR5. Such antibodies could represent research and diagnostic tools in the study of HIV infection and the development of more effective anti-HIV therapeutics. In addition, pharmaceutical compositions comprising antibodies against CCR5 may represent effective anti-HIV therapeutics.

An antibody suitable for blocking *env*-mediated membrane fusion, inhibiting chemokine binding, or blocking gp120 binding to CCR5, is specific for at least one portion of an extracellular region of the CCR5 polypeptide, as shown in Figure 1 (SEQ ID NO:2 and 4). For example, one of skill in the art can use the peptides in SEQ ID NO:5-7 or other extracellular amino acids of CCR5 to generate appropriate antibodies of the invention. Alternatively, one of skill in the art can use whole cells expressing CCR5 as an immunogen for generation of anti-CCR5 antibodies which either block *env*-mediated membrane fusion, inhibit chemokine binding or block gp120 binding to CCR5. Anti-CCR5 antibodies of the invention may have any or all of these functions.

A target cell includes but is not limited to a cell of the following types: Mv 1 Lu, NIH 3T3, BS-C-1, HEK293 cells and primary human T-cells and macrophages. Antibodies of the invention include polyclonal antibodies, monoclonal antibodies, and fragments of polyclonal and monoclonal antibodies.

The preparation of polyclonal antibodies is well-known to those skilled in the art. See, for example, Green *et al.*, *Production of Polyclonal Antisera*, in IMMUNOCHEMICAL PROTOCOLS (Manson, ed.), pages 1-5 (Humana Press 1992); Coligan *et al.*, *Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters*, in CURRENT PROTOCOLS IN IMMUNOLOGY, section 2.4.1 (1992), which are hereby incorporated by reference.

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The preparation of monoclonal antibodies likewise is conventional. See, for example, Kohler & Milstein, *Nature* 256:495 (1975); Coligan *et al.*, sections 2.5.1-2.6.7; and Harlow *et al.*, *ANTIBODIES: A LABORATORY MANUAL*, page 726 (Cold Spring Harbor Pub. 1988), which are hereby incorporated by reference. Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, e.g., Coligan *et al.*, sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes *et al.*, *Purification of Immunoglobulin G (IgG)*, in *METHODS IN MOLECULAR BIOLOGY*, VOL. 10, pages 79-104 (Humana Press 1992).

Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies is well-known to those skilled in the art. Multiplication *in vitro* may be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* may be carried out by injecting cell clones into mammals histocompatible with the parent cells, e.g., syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

Therapeutic applications for antibodies disclosed herein are also part of the present invention. For example, antibodies of the present invention may also be derived from

subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg *et al.*, International Patent Publication WO 91/11465 (1991) and Losman *et al.*, *Int. J. Cancer* 46:310 (1990), which are hereby incorporated by reference.

5 Alternatively, a therapeutically useful anti-CCR5 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:3833 (1989), which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321: 522 (1986); Riechmann *et al.*, *Nature* 332: 323 (1988); Verhoeyen *et al.*, *Science* 239: 1534 (1988); Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89: 4285 (1992); Sandhu, *Crit. Rev. Biotech.* 12: 437 (1992); and Singer *et al.*, *J. Immunol.* 150: 2844 (1993), which are hereby incorporated by reference.

10 Antibodies of the invention also may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, VOL. 2, page 119 (1991); Winter *et al.*, *Ann. Rev. Immunol.* 12: 433 (1994), which are hereby incorporated by reference. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

15 In addition, antibodies of the present invention may be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the

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endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al.*, *Nature Genet.* 7:13 (1994); Lonberg *et al.*, *Nature* 368:856 (1994); and Taylor *et al.*, *Int. Immunol.* 6:579 (1994), which are hereby incorporated by reference.

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Antibody fragments of the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulphydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patents No. 4,036,945 and No. 4,331,647, and references contained therein. These patents are hereby incorporated in their entireties by reference. See also Nisonhoff *et al.*, *Arch. Biochem. Biophys.* 89:230 (1960); Porter, *Biochem. J.* 73:119 (1959); Edelman *et al.*, METHODS IN ENZYMOLOGY, VOL. 1, page 422 (Academic Press 1967); and Coligan *et al.* at sections 2.8.1-2.8.10 and 2.10.1-2.10.4.

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Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

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For example, Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent, as described in Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA* 69:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, e.g., Sandhu, *supra*. Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an

oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow *et al.*, METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, VOL. 2, page 97 (1991); Bird *et al.*, *Science* 242:423-426 (1988); Ladner *et al.*, U.S. patent No. 4,946,778; Pack *et al.*, *Bio/Technology* 11: 1271-77 (1993); and Sandhu, *supra*.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick *et al.*, METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, VOL. 2, page 106 (1991).

Antibodies that bind to CXCR4 chemokine receptor, another HIV fusion cofactor receptor, have been shown to block fusion of HIV strains that use CXCR4 receptor for infection (Feng, *et al.*, *Science* 272:872, 1996; Endres, *et al.*, *Cell* 87:745, 1996).

VARIANTS OF CCR5

The term "CCR5 variant" as used herein means a molecule that simulates at least part of the structure of CCR5 and interferes with the fusion of cells that express *env* with cells that express CD4 and CCR5. The *env* protein of certain HIV isolates may participate in HIV infectivity by binding to CCR5 at the surface of certain cells. While not wishing to be bound by a particular theory of the invention, the inventors believe that CCR5 variants may interfere in HIV infectivity by competing with the binding of CCR5 to *env*. CCR5 variants may also be useful in preventing chemokine binding, thereby ameliorating symptoms of macrophage associated immune disorders.

In one embodiment, the present invention relates to peptides and peptide derivatives that have fewer amino acid residues than CCR5 and that block membrane fusion between HIV and a target cell. Such peptides and peptide derivatives could represent research and diagnostic tools in the study of HIV infection and the development of more effective anti-HIV

therapeutics. The preferred peptide fragments of CCR5 according to the invention include those which correspond to the regions of CCR5 that are exposed on the cell surface (e.g., SEQ ID NO:5, 6 or 7).

The invention relates not only to peptides and peptide derivatives of naturally-occurring CCR5, but also to CCR5 mutants and chemically synthesized derivatives of CCR5 that block membrane fusion between HIV and a target cell. For example, changes in the amino acid sequence of CCR5 are contemplated in the present invention. CCR5 can be altered by changing the DNA encoding the protein (e.g., SEQ ID NO:1&2). Preferably, only conservative amino acid alterations are undertaken, using amino acids that have the same or similar properties. Illustrative amino acid substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine or leucine.

Variants useful for the present invention comprise analogs, homologs, muteins and mimetics of CCR5 that retain the ability to block membrane fusion. Peptides of the CCR5 refer to portions of the amino acid sequence of CCR5 that also retain this ability. The variants can be generated directly from CCR5 itself by chemical modification, by proteolytic enzyme digestion, or by combinations thereof. Additionally, genetic engineering techniques, as well as methods of synthesizing polypeptides directly from amino acid residues, can be employed.

Peptides of the invention include the following which correspond to extracellular loops of CCR5 (amino acid designations are according to the single letter code):

25 extracellular loop-1 (el-1) (SEQ ID NO:4): A/LAAQWDFGNTMC

extracellular loop-2 (el-2) (SEQ ID NO:5): RSQKEGLHYTCSSHFPYSQYQFWK

extracellular loop-3 (el-3) (SEQ ID NO:6): QEFFGLNNCSSSNRLD

Figure 2 shows the ability of SEQ ID NO: 4, 5 and 6 to inhibit fusion between cells expressing the HIV-1 env (from the macrophage tropic Ba-L isolate) and murine cells co-expressing CD4 and CCR5.

Peptides of the invention can be synthesized by such commonly used methods as t-BOC or Fmoc protection of alpha-amino groups. Both methods involve stepwise syntheses whereby a single amino acid is added at each step starting from the C terminus of the peptide (See, Coligan, et al., *Current Protocols in Immunology*, Wiley Interscience, 1991, Unit 9). Peptides of the invention can also be synthesized by the well known solid phase peptide synthesis methods described Merrifield, *J. Am. Chem. Soc.*, 85:2149, 1962), and Stewart and Young, *Solid Phase Peptides Synthesis*, (Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

Alternatively, peptides can be produced by recombinant methods as described below.

The term "substantially purified" as used herein refers to a molecule, such as a peptide that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. One skilled in the art can purify CCR5 peptides using standard protein purification methods and the purity of the polypeptides can be determined using standard methods including, e.g., polyacrylamide gel electrophoresis (e.g., SDS-PAGE), column chromatography (e.g., high performance liquid chromatography (HPLC)), and amino-terminal amino acid sequence analysis.

Non-peptide compounds that mimic the binding and function of CCR5 ("mimetics") can be produced by the approach outlined in Saragovi et al., *Science* 253: 792-95 (1991). Mimetics are molecules which mimic elements of protein secondary structure. See, for

example, Johnson *et al.*, "Peptide Turn Mimetics," in BIOTECHNOLOGY AND PHARMACY, Pezzuto *et al.*, Eds., (Chapman and Hall, New York 1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions. For the purposes of the present invention, appropriate mimetics can be considered to be the equivalent of CCR5 itself.

Longer peptides can be produced by the "native chemical" ligation technique which links together peptides (Dawson, *et al.*, *Science*, 266:776, 1994). Variants can be created by recombinant techniques employing genomic or cDNA cloning methods. Site-specific and region-directed mutagenesis techniques can be employed. See CURRENT PROTOCOLS IN MOLECULAR BIOLOGY vol. 1, ch. 8 (Ausubel *et al.* eds., J. Wiley & Sons 1989 & Supp. 1990-93); PROTEIN ENGINEERING (Oxender & Fox eds., A. Liss, Inc. 1987). In addition, linker-scanning and PCR-mediated techniques can be employed for mutagenesis. See PCR TECHNOLOGY (Erlich ed., Stockton Press 1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, *supra*. Protein sequencing, structure and modeling approaches for use with any of the above techniques are disclosed in PROTEIN ENGINEERING, *loc. cit.*, and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, *supra*.

If the compounds described above are employed, the skilled artisan can routinely insure that such compounds are amenable for use with the present invention in view of the vaccinia cell fusion system described herein. If a compound blocks *env*-mediated membrane fusion (i) involved in HIV entry into a human CD4-positive target cell or (ii) between an HIV-infected cell and an uninfected human CD4-positive target cell, the compound is suitable according to the invention.

25 **CCR5-BINDING AND BLOCKING AGENTS**

In yet another embodiment, the present invention relates to CCR5-binding agents that block membrane fusion between HIV and a target cell. Such agents could represent research and diagnostic tools in the study of HIV infection and the development of more effective anti-HIV therapeutics. In addition, pharmaceutical compositions comprising CCR5-binding

agents may represent effective anti-HIV therapeutics. In the context of HIV infection, the phrase "CCR5-binding agent" denotes a naturally occurring ligand of CCR5 such as, for example: RANTES, MIP-1 α or MIP-1 β ; a synthetic ligand of CCR5, or appropriate derivatives of the natural or synthetic ligands. The determination and isolation of ligands is well described in the art. See, e.g., Lerner, *Trends NeuroSci.* 17:142-146 (1994) which is hereby incorporated in its entirety by reference. A CCR5-binding agent that blocks *env*-mediated membrane fusion (i) involved in HIV entry into a human CD4-positive target cell or (ii) between an HIV-infected cell and an uninfected human CD4-positive target cell is suitable according to the invention. Further, a CCR5 blocking or binding agent includes an agent which inhibits gp120 binding to CCR5 or chemokine binding to CCR5.

In yet another embodiment, the present invention relates to CCR5-binding agents that interfere with binding between CCR5 and a chemokine. Such binding agents may interfere by competitive inhibition, by non-competitive inhibition or by uncompetitive inhibition.

Interference with normal binding between CCR5 and one or more chemokines can result in a useful pharmacological effect related to inflammation because CCR5 binds chemokines that regulate monocyte accumulation and activation in inflamed tissue sites. Nevertheless, while monocyte chemotaxis is the most widely shared and perhaps best described function for MIP-1 α , MIP-1 β and RANTES, apparently each of the CC CKRs that bind one or more of these chemokines connect specifically and differentially to additional monocyte functions such as T-cell costimulation.

Monocytes are long-lived cells capable of further differentiation as they move from the blood to establish residence in the tissues as macrophages. The functional properties of tissue macrophages differ in different organs, and in the same organ depending on the presence of priming agents, i.e., agents that can change the behavior of monocytes and make them more sensitive to chemoattractants. CCR5-binding or blocking agents can interfere with the normal functioning of this system to reduce inflammation and are contemplated by the present invention. Anti-CCR5 antibodies of the invention are also useful in this context.

SCREEN FOR CCCR5 BINDING AND BLOCKING COMPOSITIONS

In another embodiment, the invention provides a method for identifying a composition which binds to CCR5 or blocks HIV *env*-mediated membrane fusion. The method includes incubating components comprising the composition and CCR5 under conditions sufficient to allow the components to interact and measuring the binding of the composition to CCR5.

Compositions that bind to CCR5 include peptides, peptidomimetics, polypeptides, chemical compounds and biologic agents as described above. In addition to inhibition of cell fusion, one of skill in the art could screen for inhibition of gp120 binding or inhibition of CCR5 binding to a chemokine to determine if a compound or composition was a CCR5 binding or blocking agent.

Incubating includes conditions which allow contact between the test composition and CCR5. Contacting includes in solution and in solid phase. The test ligand(s)/composition may optionally be a combinatorial library for screening a plurality of compositions. Compositions identified in the method of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki, *et al.*, *Bio/Technology*, 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landegren, *et al.*, *Science*, 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren, *et al.*, *Science*, 242:229-237, 1988).

To determine if a composition can functionally complex with the receptor protein, induction of the exogenous gene is monitored by monitoring changes in the protein levels of the protein encoded for by the exogenous gene, for example. When a composition(s) is found that can induce transcription of the exogenous gene, it is concluded that this composition(s) can bind to the receptor protein coded for by the nucleic acid encoding the initial sample test composition(s).

Expression of the exogenous gene can be monitored by a functional assay or assay for a protein product, for example. The exogenous gene is therefore a gene which will provide an assayable/measurable expression product in order to allow detection of expression of the exogenous gene. Such exogenous genes include, but are not limited to, reporter genes such

as chloramphenicol acetyltransferase gene, an alkaline phosphatase gene, beta-galactosidase, a luciferase gene, a green fluorescent protein gene, guanine xanthine phosphoribosyltransferase, alkaline phosphatase, and antibiotic resistance genes (e.g., neomycin phosphotransferase).

Expression of the exogenous gene is indicative of composition-receptor binding, thus. 5 the binding or blocking composition can be identified and isolated. The compositions of the present invention can be extracted and purified from the culture media or a cell by using known protein purification techniques commonly employed, such as extraction, precipitation, ion exchange chromatography, affinity chromatography, gel filtration and the like. Compositions can be isolated by affinity chromatography using the modified receptor protein 10 extracellular domain bound to a column matrix or by heparin chromatography.

Also included in the screening method of the invention is combinatorial chemistry methods for identifying chemical compounds that bind to CCR5. Ligands/compositions that bind to CCR5 can be assayed in standard cell:cell fusion assays, such as the vaccinia assay described herein to determine whether the composition inhibits or blocks *env*-mediated 15 membrane fusion (i) involved in HIV entry into a human CD4-positive target cell or (ii) between an HIV-infected cell and an uninfected human CD4-positive target cell. Screening methods include inhibition of chemokine binding to CCR5 (e.g., use radiolabeled chemokine) or inhibition of labeled gp120. For example, a derivative of RANTES was shown to act as a CCR5 receptor antagonist (RANTES 9-68; Arenzana-Selsdedos et al., *Nature* 383:400, 1996, 20 incorporated by reference). AOP-RANTES and Met-RANTES were shown to bind with high affinity yet failed to induce chemotaxis signalling, thereby acting as an antagonist (Simmons et al., *Science* 276:276, 1997). Thus, the screening method is also useful for identifying variants, binding or blocking agents, etc., which functionally, if not physically (e.g., sterically) act as antagonists or agonists, as desired.

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PHARMACEUTICAL COMPOSITIONS

The invention also includes various pharmaceutical compositions that block membrane fusion between HIV and a target cell. The pharmaceutical compositions according to the invention are prepared by bringing an antibody against CCR5, a peptide or peptide derivative of CCR5, a CCR5 mimetic, or a CCR5-binding agent according to the present invention into

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a form suitable for administration to a subject using carriers, excipients and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described, for instance, in *Remington's Pharmaceutical Sciences*, 15th ed. Easton: Mack Publishing Co., 1405-1412, 1461-1487 (1975) and *The National Formulary XIV.*, 14th ed. Washington: American Pharmaceutical Association (1975), the contents of which are hereby incorporated by reference. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art. *See Goodman and Gilman's The Pharmacological Basis for Therapeutics* (7th ed.).

In another embodiment, the invention relates to a method of blocking the membrane fusion between HIV and a target cell. This method involves administering to a subject a therapeutically effective dose of a pharmaceutical composition containing the compounds of the present invention and a pharmaceutically acceptable carrier. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. By "subject" is meant any mammal, preferably a human.

The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patient, different daily doses are necessary. Under certain circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The pharmaceutical compositions according to the invention are in general administered topically, intravenously, orally or parenterally or as implants, but even rectal use

is possible in principle. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science*, 249: 1527-1533 (1990), which is incorporated herein by reference.

The pharmaceutical compositions according to the invention may be administered locally or systemically. By "therapeutically effective dose" is meant the quantity of a compound according to the invention necessary to prevent, to cure or at least partially arrest the symptoms of the disease and its complications. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the patient. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, e.g., in Gilman *et al.* (eds.) (1990) GOODMAN AND GILMAN'S: THE PHARMACOLOGICAL BASES OF THERAPEUTICS, 8th ed., Pergamon Press; and REMINGTON'S PHARMACEUTICAL SCIENCES, 17th ed. (1990), Mack Publishing Co., Easton, Pa., each of which is herein incorporated by reference.

TESTING FOR NEW PHARMACEUTICAL COMPOSITIONS

In a preferred embodiment, the invention is a method for screening a compound ("test substance") for anti-HIV pharmacological activity. In this embodiment, the CCR5 and CD4 genes are expressed in one type of eukaryotic cell and incubated with a second type of eukaryotic cell that expresses an HIV envelope protein ("env"). Fusion between at least one cell of each type with the other type is then monitored. The test substance is added to the incubation solution before or after mixing of the cells and its effect on the fusion rate of cells is determined by any of a number of means. One means to monitor fusion is to include a

system that results in the production of an active β -galactosidase upon cell fusion as described in Nussbaum et al., 1994, *supra*. If the test molecule inhibits HIV infectivity then the presence of the molecule will decrease the cell fusion response. In the case where the test substance binds a naturally occurring molecule present in the human that is necessary for HIV infectivity, then addition of the test molecule may decrease cell fusion.

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The cell fusion assay can be used to determine the functional ability of CCR5 to confer *env*-mediated fusion competence to a diverse range of CD4-positive (e.g., either recombinantly produced or naturally occurring) cell types: e.g., NIH 3T3 (murine); BS-C-1 (African green monkey); HEK293 (human); and Mv 1 Lu (mink). In addition, unusual, fusion-incompetent, CD4-positive human cell types can be employed (U-87 MG glioblastoma; and SCL1).

Variations of drug screening methods are known to the artisan of average skill in this field. Consequently, the cell fusion assay can be used in a wide variety of formats to exploit the properties of the CCR5 receptor to screen for drugs that are effective against HIV.

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ANTISENSE or RIBOZYME INHIBITION OF CCR5 FOR HIV THERAPY

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Antisense technology offers a very specific and potent means of inhibiting HIV infection of cells that contain CCR5, for example, by decreasing the amount of CCR5 expression in a cell. Antisense polynucleotides in context of the present invention includes both short sequences of DNA known as oligonucleotides of usually 10-50 bases in length as well as longer sequences of DNA that may exceed the length of the CCR5 gene sequence itself. Antisense polynucleotides useful for the present invention are complementary to specific regions of a corresponding target mRNA. Hybridization of antisense polynucleotides to their target transcripts can be highly specific as a result of complementary base pairing. The capability of antisense polynucleotides to hybridize is affected by such parameters as length, chemical modification and secondary structure of the transcript which can influence polynucleotide access to the target site. See Stein et al, *Cancer Research* 48:2659 (1988). An antisense polynucleotide can be introduced to a cell by introducing a DNA segment that codes for the polynucleotide into the cell such that the polynucleotide is made inside the cell. An antisense polynucleotide can also be introduced to a cell by adding the polynucleotide to the

environment of the cell such that the cell can take up the polynucleotide directly. The latter route is preferred for the shorter polynucleotides of up to about 20 bases in length.

In selecting the preferred length for a given polynucleotide, a balance must be struck to gain the most favorable characteristics. Shorter polynucleotides such as 10-to 15-mers, while offering higher cell penetration, have lower gene specificity. In contrast, while longer polynucleotides of 20-30 bases offer better specificity, they show decreased uptake kinetics into cells. See Stein *et al.*, PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDE ANALOGUES in "Oligodeoxynucleotides - Antisense Inhibitors of Gene Expression" Cohen, ed. McMillan Press, London (1988). Accessibility to mRNA target sequences also is of importance and, therefore, loop-forming regions in targeted mRNAs offer promising targets.

In this disclosure the term "polynucleotide" encompasses both oligomeric nucleic acid moieties of the type found in nature, such as the deoxyribonucleotide and ribonucleotide structures of DNA and RNA, and man-made analogues which are capable of binding to nucleic acids found in nature. The polynucleotides of the present invention can be based upon ribonucleotide or deoxyribonucleotide monomers linked by phosphodiester bonds, or by analogues linked by methyl phosphonate, phosphorothioate, or other bonds. They may also comprise monomer moieties which have altered base structures or other modifications, but which still retain the ability to bind to naturally occurring DNA and RNA structures. Such polynucleotides may be prepared by methods well-known in the art, for instance using commercially available machines and reagents available from Perkin-Elmer/Applied Biosystems (Foster City, CA).

Phosphodiester-linked polynucleotides are particularly susceptible to the action of nucleases in serum or inside cells, and therefore in a preferred embodiment the polynucleotides of the present invention are phosphorothioate or methyl phosphonate-linked analogues, which have been shown to be nuclease-resistant. Persons of ordinary skill in this art will be able to select other linkages for use in the invention. These modifications also may be designed to improve the cellular uptake and stability of the polynucleotides.

In another embodiment of the invention, the antisense polynucleotide is an RNA molecule produced by introducing an expression construct into the target cell. The RNA molecule thus produced is chosen to have the capability to hybridize to CCR5 mRNA. Such

molecules that have this capability can inhibit translation of the CCR5 mRNA and thereby inhibit the ability of HIV to infect cells that contain the RNA molecule.

The polynucleotides which have the capability to hybridize with mRNA targets can inhibit expression of corresponding gene products by multiple mechanisms. In "translation arrest," the interaction of polynucleotides with target mRNA blocks the action of the ribosomal complex and, hence, prevents translation of the messenger RNA into protein. Haeuptle *et al.*, *Nucl. Acids. Res.* 14:1427 (1986). In the case of phosphodiester or phosphorothioate DNA polynucleotides, intracellular RNase H can digest the targeted RNA sequence once it has hybridized to the DNA oligomer. Walder and Walder, *Proc. Natl. Acad. Sci. USA* 85:5011 (1988). As a further mechanism of action, in "transcription arrest" it appears that some polynucleotides can form "triplex," or triple-helical structures with double stranded genomic DNA containing the gene of interest, thus interfering with transcription by RNA polymerase. Giovannangeli *et al.*, *Proc. Natl. Acad. Sci.* 90:10013 (1993); Ebbinghaus *et al.* *J. Clin. Invest.* 92:2433 (1993).

In one preferred embodiment, CCR5 polynucleotides are synthesized according to standard methodology. Phosphorothioate modified DNA polynucleotides typically are synthesized on automated DNA synthesizers available from a variety of manufacturers. These instruments are capable of synthesizing nanomole amounts of polynucleotides as long as 100 nucleotides. Shorter polynucleotides synthesized by modern instruments are often suitable for use without further purification. If necessary, polynucleotides may be purified by polyacrylamide gel electrophoresis or reverse phase chromatography. See Sambrook *et al.*, *MOLECULAR CLONING: A Laboratory Manual*, Vol. 2, Chapter 11, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Alternatively, a CCR5 polynucleotide in the form of antisense RNA may be introduced to a cell by its expression within the cell from a standard DNA expression vector. CCR5 DNA antisense sequences can be cloned from standard plasmids into expression vectors, which expression vectors have characteristics permitting higher levels of, or more efficient expression of the resident polynucleotides. At a minimum, these constructs require a prokaryotic or eukaryotic promoter sequence which initiates transcription of the inserted DNA sequences. A preferred expression vector is one where the expression is inducible to high

levels. This is accomplished by the addition of a regulatory region which provides increased transcription of downstream sequences in the appropriate host cell. See Sambrook *et al.*, Vol. 3, Chapter 16 (1989).

For example, CCR5 antisense expression vectors can be constructed using the polymerase chain reaction (PCR) to amplify appropriate fragments from single-stranded cDNA of a plasmid such as pRc in which CCR5 cDNA has been incorporated. Fang *et al.*, *J. Biol. Chem.* **267** 25889-25897 (1992). Polynucleotide synthesis and purification techniques are described in Sambrook *et al.* and Ausubel *et al.* (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley Interscience 1987) (hereafter "Ausubel"), respectively. The PCR procedure is performed via well-known methodology. See, for example, Ausubel, and Bangham, "The Polymerase Chain Reaction: Getting Started," in PROTOCOLS IN HUMAN MOLECULAR GENETICS (Humana Press 1991). Moreover, PCR kits can be purchased from companies such as Stratagene Cloning Systems (La Jolla, CA) and Invitrogen (San Diego, CA).

The products of PCR are subcloned into cloning vectors. In this context, a "cloning vector" is a DNA molecule, such as a plasmid, cosmid or bacteriophage, that can replicate autonomously in a host prokaryotic cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Suitable cloning vectors are described by Sambrook *et al.*, Ausubel, and Brown (ed.), MOLECULAR BIOLOGY LABFAX (Academic Press 1991). Cloning vectors can be obtained, for example, from GIBCO/BRL (Gaithersburg, MD), Clontech Laboratories, Inc. (Palo Alto, CA), Promega Corporation (Madison, WI), Stratagene Cloning Systems (La Jolla, CA), Invitrogen (San Diego, CA), and the American Type Culture Collection (Rockville, MD).

Preferably, the PCR products are ligated into a "TA" cloning vector. Methods for generating PCR products with a thymidine or adenine overhang are well-known to those of skill in the art. See, for example, Ausubel at pages 15.7.1-15.7.6. Moreover, kits for

performing TA cloning can be purchased from companies such as Invitrogen (San Diego, CA).

Cloned antisense fragments are amplified by transforming competent bacterial cells with a cloning vector and growing the bacterial host cells in the presence of the appropriate antibiotic. See, for example, Sambrook *et al.*, and Ausubel. PCR is then used to screen bacterial host cells for CCR5 antisense orientation clones. The use of PCR for bacterial host cells is described, for example, by Hofmann *et al.*, "Sequencing DNA Amplified Directly from a Bacterial Colony," in PCR PROTOCOLS: METHODS AND APPLICATIONS, White (ed.), pages 205-210 (Humana Press 1993), and by Cooper *et al.*, "PCR-Based Full-Length cDNA Cloning Utilizing the Universal-Adaptor/Specific DOS Primer-Pair Strategy," *Id.* at pages 305-316.

Cloned antisense fragments are cleaved from the cloning vector and inserted into an expression vector. For example, *Hind*III and *Xba*I can be used to cleave the antisense fragment from TA cloning vector pCRTM-II (Invitrogen; San Diego, CA). Suitable expression vectors typically contain (1) prokaryotic DNA elements coding for a bacterial origin of replication and an antibiotic resistance marker to provide for the amplification and selection of the expression vector in a bacterial host; (2) DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence.

For a mammalian host, the transcriptional and translational regulatory signals preferably are derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as actin, collagen, myosin, and metallothionein genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer *et al.*, *J. Molec. Appl. Genet.* 1: 273 (1982)); the TK promoter of Herpes virus (McKnight, *Cell* 31: 355 (1982)); the SV40 early promoter (Benoist *et al.*, *Nature* 290: 304 (1981); the Rous sarcoma virus promoter (Gorman *et al.*, *Proc. Nat'l Acad.*

Sci. USA 79: 6777 (1982)); and the cytomegalovirus promoter (Foecking *et al.*, Gene 45: 101 (1980)).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control fusion gene expression if the prokaryotic promoter is regulated by a eukaryotic promoter. Zhou *et al.*, Mol. Cell. Biol. 10: 4529 (1990); Kaufman *et al.*, Nucl. Acids Res. 19: 4485 (1991).

A vector for introducing at least one antisense polynucleotide into a cell by expression from a DNA is the vector pRc/CMV (Invitrogen (San Diego, CA), which provides a high level of constitutive transcription from mammalian enhancer-promoter sequences. Cloned CCR5 antisense vectors are amplified in bacterial host cells, isolated from the cells, and analyzed as described above.

Another possible method by which antisense sequences may be exploited is via gene therapy. Virus-like vectors, usually derived from retroviruses, may prove useful as vehicles for the importation and expression of antisense constructs in human cells. Generally, such vectors are non-replicative *in vivo*, precluding any unintended infection of non-target cells. In such cases, helper cell lines are provided which supply the missing replicative functions *in vitro*, thereby permitting amplification and packaging of the antisense vector. A further precaution against accidental infection of non-target cells involves the use of target cell-specific regulatory sequences. When under the control of such sequences, antisense constructs would not be expressed in normal tissues.

Two prior studies have explored the feasibility of using antisense polynucleotides to inhibit the expression of a heparin binding growth factor. Kouhara *et al.*, Oncogene 9: 455-462 (1994); Morrison, J. Biol. Chem. 266: 728 (1991). Kouhara *et al.* showed that androgen-dependent growth of mouse mammary carcinoma cells (SC-3) is mediated through induction of androgen-induced, heparin binding growth factor (AIGF). An antisense 15-mer corresponding to the translation initiation site of AIGF was measured for its ability to interfere with androgen-induction of SC-3 cells. At concentrations of 5 µM, the antisense polynucleotide effectively inhibited androgen-induced DNA synthesis. Morrison showed that antisense polynucleotides targeted against basic fibroblast growth factor can inhibit growth

of astrocytes in culture. Thus, the general feasibility of targeting an individual gene product in a mammalian cell has been established.

5 Antisense polynucleotides according to the present invention are derived from any portion of the open reading frame of the CCR5 cDNA. Preferably, mRNA sequences (i) surrounding the translation initiation site and (ii) forming loop structures are targeted. Based upon the size of the human genome, statistical studies show that a DNA segment approximately 14-15 base pairs long will have a unique sequence in the genome. To ensure specificity of targeting CCR5 RNA, therefore, it is preferred that the antisense polynucleotides are at least 15 nucleotides in length. Thus, the shortest polynucleotides contemplated by the 10 present invention encompass nucleotides corresponding to positions 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 2-16, 3-17, etc. of the CCR5 cDNA sequence. Position 1 refers to the first nucleotide of the CCR5 coding region.

15 Not every antisense polynucleotide will provide a sufficient degree of inhibition or a sufficient level of specificity for the CCR5 target. Thus, it will be necessary to screen polynucleotides to determine which have the proper antisense characteristics. A preferred method to assay for a useful antisense polynucleotide is the inhibition of cell fusion between: (1) cells that contain CD4 and CCR5; and (2) cells that contain env.

20 Administration of an antisense polynucleotide to a subject, either as a naked, synthetic polynucleotide or as part of an expression vector, can be effected via any common route (oral, nasal, buccal, rectal, vaginal, or topical), or by subcutaneous, intramuscular, intraperitoneal, or intravenous injection. Pharmaceutical compositions of the present invention, however, are advantageously administered in the form of injectable compositions. A typical composition for such purpose comprises a pharmaceutically acceptable solvent or diluent and other suitable, physiologic compounds. For instance, the composition may contain polynucleotide 25 and about 10 mg of human serum albumin per milliliter of a phosphate buffer containing NaCl.

30 As much as 700 milligrams of antisense polynucleotide has been administered intravenously to a patient over a course of 10 days (*i.e.*, 0.05 mg/kg/hour) without signs of toxicity. Sterling, "Systemic Antisense Treatment Reported," *Genetic Engineering News* 12: 1, 28 (1992).

Other pharmaceutically acceptable excipients include non-aqueous or aqueous solutions and non-toxic compositions including salts, preservatives, buffers and the like. Examples of non-aqueous solutions are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyoleate. Aqueous solutions include water, 5 alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride. Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to routine skills in the art. A preferred pharmaceutical composition for 10 topical administration is a dermal cream or transdermal patch.

Antisense polynucleotides or their expression vectors may be administered by injection as an oily suspension. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides. Moreover, antisense polynucleotides or vectors may be combined with a lipophilic carrier such as any 15 one of a number of sterols including cholesterol, cholate and deoxycholic acid. A preferred sterol is cholesterol. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension also contains stabilizers.

An alternative formulation for the administration of antisense CCR5 polynucleotides 20 involves liposomes. Liposome encapsulation provides an alternative formulation for the administration of antisense CCR5 polynucleotides and expression vectors. Liposomes are microscopic vesicles that consist of one or more lipid bilayers surrounding aqueous compartments. See, generally, Bakker-Woudenberg *et al.*, *Eur. J. Clin. Microbiol. Infect. Dis.* 12 (Suppl. 1): S61 (1993), and Kim, *Drugs* 46: 618 (1993). Liposomes are similar in 25 composition to cellular membranes and as a result, liposomes can be administered safely and are biodegradable. Depending on the method of preparation, liposomes may be unilamellar or multilamellar, and liposomes can vary in size with diameters ranging from 0.02 µm to greater than 10 µm. A variety of agents can be encapsulated in liposomes: hydrophobic agents partition in the bilayers and hydrophilic agents partition within the inner aqueous space(s). 30 See, for example, Machy *et al.*, LIPOSOMES IN CELL BIOLOGY AND

PHARMACOLOGY (John Libbey 1987), and Ostro *et al.*, *American J. Hosp. Pharm.* **46**: 1576 (1989). Moreover, it is possible to control the therapeutic availability of the encapsulated agent by varying liposome size, the number of bilayers, lipid composition, as well as the charge and surface characteristics of the liposomes.

5 Liposomes can adsorb to virtually any type of cell and then slowly release the encapsulated agent. Alternatively, an absorbed liposome may be endocytosed by cells that are phagocytic. Endocytosis is followed by intralysosomal degradation of liposomal lipids and release of the encapsulated agents. Scherphof *et al.*, *Ann. N.Y. Acad. Sci.* **446**: 368 (1985).

10 After intravenous administration, conventional liposomes are preferentially phagocytosed into the reticuloendothelial system. However, the reticuloendothelial system can be circumvented by several methods including saturation with large doses of liposome particles, or selective macrophage inactivation by pharmacological means. Claassen *et al.*, *Biochim. Biophys. Acta* **802**: 428 (1984). In addition, incorporation of glycolipid- or polyethelene glycol-derivatised phospholipids into liposome membranes has been shown to result in a significantly reduced uptake by the reticuloendothelial system. Allen *et al.*, *Biochim. Biophys. Acta* **1068**: 133 (1991); Allen *et al.*, *Biochim. Biophys. Acta* **1150**: 9 (1993). These Stealth® liposomes have an increased circulation time and an improved targeting to tumors in animals. Woodle *et al.*, *Proc. Amer. Assoc. Cancer Res.* **33**: 2672 (1992). Human clinical trials are in progress, including Phase III clinical trials against Kaposi's sarcoma. Gregoriadis *et al.*, *Drugs* **45**: 15 (1993).

15 20 Antisense polynucleotides and expression vectors can be encapsulated within liposomes using standard techniques. A variety of different liposome compositions and methods for synthesis are known to those of skill in the art. See, for example, U.S. Patent No. 4,844,904, U.S. Patent No. 5,000,959, U.S. Patent No. 4,863,740, and U.S. Patent No. 4,975,282, all of which are hereby incorporated by reference.

25 Liposomes can be prepared for targeting to particular cells or organs by varying phospholipid composition or by inserting receptors or ligands into the liposomes. For instance, antibodies specific to tumor associated antigens may be incorporated into liposomes, together with antisense polynucleotides or expression vectors, to target the liposome more effectively to the tumor cells. See, for example, Zelphati *et al.*, *Antisense Research and*

Development 3: 323-338 (1993), describing the use "immunoliposomes" containing antisense polynucleotides for human therapy.

In general, the dosage of administered liposome-encapsulated antisense polynucleotides and vectors will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Dose ranges for particular formulations can be determined by using a suitable animal model.

The above approaches can also be used not only with antisense nucleic acid, but also with ribozymes, or triplex agents to block transcription or translation of a specific CCR5 mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme.

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, *et al.*, *Antisense Res. and Dev.*, 1(3):227, 1991; Helene, C., *Anticancer Drug Design*, 6(6):569, 1991).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, *J.Amer.Med. Assn.*, 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, *Nature*, 334:585, 1988) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

HOMOZYGOUS AND HETEROZYGOUS MUTATIONS IN CCR5

It is known that in some cases, a homozygous or heterozygous mutation in a polypeptide or a regulatory region of a gene confers a molecular basis for a difference in function. Bertina, *et al.* and Greengard, *et al.* (Bertina, *et al.*, *Nature*, 369:64, 1994; Greengard, *et al.*, *Lancet*, 343:1361, 1994), first identified the molecular basis for the FV abnormality. The phenotype of APC resistance was shown to be associated with heterozygosity or homozygosity for a single point mutation in the FV gene that resulted in the substitution of arginine at amino acid residue 506 with glutamine (FV R506Q). This R506Q mutation prevents APC from cleaving a peptide bond at Arg-506 in FV that is required to inactivate factor Va (Bertina, *supra*; Sun, *et al.*, *Blood*, 83:3120, 1994).

Similarly, the present invention envisions diagnostic and prognostic, and in addition, therapeutic approaches to treatment of HIV-associated syndromes based on homozygosity or heterozygosity of CCR5 mutants. For example, while not wanting to be bound by a particular theory, it is believed that a subject having a homozygous mutant of CCR5 may be HIV resistant or exhibit a slower rate of disease progression. Along the same lines, a subject having a heterozygous mutation in CCR5 may exhibit a slower rate of disease progression than a patient having a wild type CCR5. Mutations included in the CCR5 coding region may also result in inactivating mutations. In addition, a mutation in the regulatory region of CCR5 gene may prevent or inhibit expression of CCR5, thereby providing resistance to some degree from HIV infection.

Once an individual having a homozygous or heterozygous mutant in CCR5 is identified, it is envisioned that cells from that individual, once matched for histocompatibility, can be transplanted to an HIV positive individual, or to an "at risk" individual.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are to be considered illustrative and thus are not limiting of the remainder of the disclosure in any way whatsoever.

Example 1. Cloning and Sequence Analysis of the CCR5 Gene

Complementary DNAs were obtained from a λ gt11 cDNA library (Combadiere *et al.*, *DNA Cell Biol. cit op.*). One of the cDNAs obtained, designated clone 63-2, had a novel sequence highly related to CC CKR2B, but it extended only from bp 105 to 813 of the CC CKR2B open reading frame. The 63-2 cDNA was used as a hybridization probe to screen under low stringency conditions (final wash in 5X SSPE; described by Maniatis *et al.*, Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory, at 55°C for 30 min) a λ pCEV9 cDNA library prepared from endotoxin-stimulated human peripheral blood monocytes as described previously (Combadiere *et al.*, *J. Biol. Chem.* 270: 29671-5 and *J. Biol. Chem.* 270: 16491-4 (1995)). One of the isolated clones, designated clone 8.5, matched and extended the sequence of clone 63-2. A 1.4 kb fragment of the clone 8.5 cDNA was excised from the vector DNA by *Bam* HI and *Bst* XI double digestion, blunt-ended with *Pfu* DNA polymerase, subcloned into the *Eco* RV site of pBluescript II KS (Stratagene, La Jolla, CA), and sequenced completely on both strands. The cDNA insert was then subcloned between the *Bam* HI and *Hind* III sites of the mammalian expression vector pREP9 (Invitrogen, San Diego, CA).

The clone 8.5 cDNA is 1.4 kb in length. The 5'- and 3'-untranslated regions and open reading frame are 26,288 and 1056 bp, respectively. The 3'-untranslated region is not polyadenylated and lacks a polyadenylation consensus sequence. The ATG codon proposed to initiate translation is flanked by sequence that conforms favorably with established consensus rules. M. Kozak, *Nucleic Acids Res.* 15: 8125-48 (1987). The open reading frame contains 352 codons.

Seven segments of the deduced amino acid sequence from SEQ ID NO: 1 have a high content of hydrophobic amino acids consistent with membrane-spanning domains as well as multiple amino acids conserved in analogous positions of the known seven-transmembrane-domain receptor rhodopsin. These considerations clearly indicate that CCR5 is ancestrally related to rhodopsin-like receptors, and strongly suggest that it functions as a seven-transmembrane-domain G protein-coupled receptor. A database search revealed that the highest sequence identity occurs with chemokine receptors. In particular, the amino acid sequence of CCR5 is 57, 70, 75, 51 and 48% identical to CC CKR1, CC CKR2A, CC CKR2B,

CC CKR3 and CC CKR4, respectively, with lower identity (approximately 30%) to the CXC chemokine receptors, IL-8 receptors A and B. An alignment of the amino acid sequence of CCR5 ["SEQ ID NO: 2"] with those of CC CKR1 and CC CKR2B is shown in Figure 1.

The CC CKR2B sequence is eight amino acids longer than CCR5 due to a substantially longer N-terminal segment. The two sequences are otherwise colinear with the exception of a four amino acid gap in the putative second extracellular loop of CC CKR2B relative to that of CCR5.

Residues of CCR5 that differ from CC CKR2B in the alignment are found mostly in the putative extracellular domains (the N terminal segment and the sequence between putative transmembrane domains 2 and 3, 4 and 5, and 6 and 7) and adjacent portions of the transmembrane domains, and in the C-terminal segment which is predicted to lie in the cytoplasm (Figure 2). Like other seven-transmembrane-domain receptors, the C-terminal tail has a high content of serine and threonine residues that may be sites for receptor phosphorylation as they are in rhodopsin and the $\beta 2$ adrenergic receptor. CCR5 also contains cysteine residues that by analogy with other seven-transmembrane domain receptors could be a site for palmitoylation, tethering this domain to the plasma membrane.

The net charge of the N-terminal extracellular segment of CCR5 is -1. The corresponding domain of CC CKR2B has a net charge of zero, whereas for other known chemokine receptors this domain is highly acidic. Like all other known chemokine receptors, CCR5 has conserved cysteine residues in the N-terminal segment and the third predicted extracellular loop that could form a disulfide bond. Cysteine residues are less frequently found in this location in other seven-transmembrane-domain receptors. CCR5 lacks a consensus sequence for N-linked glycosylation, whereas one or more are found in other CC CKR's.

The CCR5 Gene. When total human genomic DNA was digested with *Pst* I, *Eco* RI, *Hind* III and *Xba* I and hybridized with a CCR5 cDNA probe extending from bp 69 to 789 relative to the start of the open reading frame, 2-4 bands that hybridized with different intensity were detected in each lane. The probe used contains a recognition site for *Pst* I at bp 532 of the CCR5 open reading frame but not for the other restriction enzymes used. The pattern is most

consistent with multiple small cross-hybridizing genes. In fact, restriction fragments of genomic clones isolated for CC CKR2, CC CKR3 and CCR5 can account for all of the bands seen. The CC CKR2B, CC CKR3 and CCR5 open reading frames lack intervening sequences.

Example 2. Chemokine Binding to CCR5

5 Human embryonic kidney (HEK) 293 cells (a total of 10^7) grown to log phase in DMEM and 10% fetal bovine serum were electroporated with 20 µg of plasmid DNA, and G418-resistant colonies were picked and expanded as described previously (Combadiere *et al.*, *op cit.*). The cell lines studied contained large amounts of the recombinant CCR5 mRNA, but lacked CC CKR3 mRNA and native 8.5 mRNA, as assessed by Northern blot analysis of
10 total RNA using full-length cDNA probes. The methods used to create HEK 293 cell lines stably expressing CC CKR1 and CC CKR2B have been described previously (Combadiere *et al.*, *J. Biol. Chem.* 270: 29671-5).

15 Transfected HEK 293 cells (a total of 10^6) were incubated in duplicate with 0.2 nM ^{125}I -labeled RANTES, MCP-1, MIP-1 α , MIP-1 β or MCP-3 (specific activity -2200 Ci/mmol, Du Pont/NEN, Boston, MA) and varying concentrations of unlabeled recombinant human chemokines (Peprotech, Rocky Hill, NJ) in 200 µl of binding medium (RPMI 1640 with 1 mg/ml BSA and 25 mM HEPES, pH 7.4). After incubation for 1 h at 4°C or 37°C, unbound chemokines were separated from cells by pelleting through a 10% sucrose/PBS cushion, and the cell-associated counts were determined. Specific binding was determined by the
20 difference in counts in the presence and absence of 1250-fold molar excess of unlabeled chemokine.

Receptor activation from chemokine binding was assessed by real time measurement of $[\text{Ca}^{2+}]_i$ changes using 2 million transfected HEK 293 cells loaded with FURA-2. Ratio fluorescence of cells was measured as described previously. Combadiere *et al.*, *J. Biol. Chem.* 270: 16491-4 (1995). J. Van Damme (Rega Institute, Leuven) provided chemically synthesized human MCP-2 protein according to a procedure described in Proost *et al.*, *Cytokine* 7: 97-104 (1995). O. Yoshie (Shionogi Institute, Osaka) provided recombinant human eotaxin. Where indicated, cells loaded with FURA-2 were incubated in holotoxin of

B. pertussis (List, Campbell, CA) 250 ng/ml for 2 h at 37°C, then washed twice in PBS and resuspended in HBSS. Cell viability was ~80% by trypan blue exclusion after pertussis toxin treatment. ATP was purchased from Sigma Co. (St. Louis, MO).

5 *Agonists for CCR5.* Given the high sequence similarity of CCR5 with other CC chemokine receptors, the inventors predicted that CCR5 would be specific for CC chemokines. To test this, the inventors transfected HEK 293 cells, which normally are unresponsive to stimulation with chemokines, with the clone 8.5 cDNA and measured the calcium flux responses induced by a panel of chemokines. The calcium flux response is strongly associated with chemotaxis, degranulation and other higher order leukocyte responses to chemokines, and is a sensitive and specific measure of receptor activation. Neither untransfected nor mock-transfected and selected HEK 293 cells responded to any of the chemokines tested. In contrast, six independent HEK 293 cell lines stably transfected with the clone 8.5 CDNA, three each from two separate transfections, exhibited [Ca²⁺]_i transients in response to MIP-1 α , RANTES and MIP-1 β , but not in response to MCP-1, MCP-2, MCP-3, eotaxin, IL-8 or γ IP-10 all tested at 100 nM. MIP-1 α , MIP-1 β and RANTES were similar in potency and efficacy, the concentrations for half-maximal and maximal responses ranging from 5-40 and 25-50 nM, respectively. These results indicate that CCR5 is a CC chemokine receptor selective for MIP-1 α , MIP-1 β and RANTES.

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20 *Desensitization of CCR5.* After activation, chemokine receptors have altered sensitivity to repeated stimulation with the activating agonist and other agonists. When the same chemokine was added in succession to CCR5 transfectants, cells responded to the first addition but not the second, indicating that the receptor underwent homologous desensitization to all three of its agonists. When different agonists were added in succession, MIP-1 α or RANTES given first blocked the response to MIP-1 β given second, and MIP-1 β or RANTES given first blocked the response to MIP-1 α given second. But MIP-1 α or MIP-1 β given first reduced, but did not eliminate, the response to RANTES given second. MCP-1 had no effect on the responses to MIP-1 α , MIP-1 β or RANTES. These data show a functional interaction of MIP-1 α , MIP-1 β and RANTES with the same receptor, CCR5.

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5 *G Protein Coupling to CCR5.* Known chemokine receptors couple to G_i-type G proteins, which unlike other classes of G proteins are functionally sensitive to pertussis toxin. Treatment of CCR5 transfectants with pertussis toxin completely abolished the calcium flux response to MIP-1 α , MIP-1 β and RANTES. In contrast, the calcium flux response to ATP was largely unaffected. These data indicate that CCR5 in HEK 293 cells is coupled to G proteins of the G_i class.

10 *Binding of CC Chemokines to CCR5.* The calcium flux results show that cells expressing CCR5 have acquired the capacity to respond to the presence of MIP-1 α , MIP-1 β and RANTES. The mechanism of this effect appears to be related to specific binding to CCR5 on the cell surfaces as judged by radioligand binding assays with intact HEK 293 cells that were stably transfected with CCR5 and by comparison with HEK 293 cells stably transfected with CC CKR1 and CC CKR2B as positive and negative controls, respectively. The results for CCR5 are quite complex, and the results for the positive and negative controls are described first.

15 The total amounts of 125 I-MIP-1 α , 125 I-MIP-1 β and 125 I-RANTES that bound to CC CKR2B were similar in magnitude to untransfected HEK 293 cells, and were completely non-specific in both cases, whether the assays were carried out at 4°C or 37°C. In contrast, CC CKR2B transfectants specifically bound both 125 I-MCP-1 and 125 I-MCP-3 at both 4°C and 37°C. These results are consistent with the known agonists for CC CKR2B.

20 In the case of CC CKR1, specific binding of 125 I-MIP-1 α was 5-10-fold greater than non-specific binding at both 4°C and 37°C, whereas specific binding of 125 I-MCP-1 was not detectable. The K_d for homologous competition binding of 125 I-MIP-1 α at 4°C was 10 nM. These results are similar to previously published results, are consistent with MIP-1 α 's agonist activity for CC CKR1, and represent a positive control for MIP-1 α binding. Each of the 25 six CCR5 transfectants were tested at 4°C. The total binding of 125 I-MIP-1 α , 125 I-MIP-1 β and 125 I-RANTES was equal to the background levels established for the CC CKR2B transfectant, and was completely non-specific even when radioligand concentrations as high as 5 nM were tested. Yet, all six cell lines exhibited clear and robust calcium flux responses to MIP-1 α , MIP-1 β and RANTES.

The G418 concentration in the media of the CCR5 transfectant that exhibited the strongest calcium flux response was increased from 1 to 3 mg/ml for one week. A cell line was derived, named CCR5.1 that exhibited calcium flux responses to MIP-1 α , MIP-1 β and RANTES that were consistently double those of the parental cell line. This cell line exhibited specific binding at 4°C for MIP-1 α , MIP-1 β and RANTES.

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To increase the sensitivity of the binding assay, non-equilibrium conditions at 37°C were used to increase the ratio of specific to non-specific binding for 125 I-MIP-1 α , 125 I-MIP-1 β and 125 I-RANTES by a factor of 2-4 for both CC CKR1 and CCR5.1 cell lines, compared to results obtained at 4°C. 125 I-MCP-1 did not bind specifically to either CC CKR1 or CCR5.1 cells at 37°C, whereas 125 I-MCP-3 bound specifically to CC CKR1 but not to CCR5.1 cells.

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The 125 I-MIP-1 α and 125 I-MIP-1 β binding sites on CC CKR1 and CCR5.1 cells were easily distinguished in two ways. First, 125 I-MIP-1 α and 125 I-MIP-1 β binding to CC CKR1, but not to CCR5.1, was competed effectively by unlabeled MCP-3. Second, unlabeled MIP-1 α competed 20-fold more effectively for 125 I-MIP-1 α binding to CC CKR1 than to CCR5.1 (half-maximal inhibitory concentrations IC_{50}) -5 and 100 nM, respectively), and unlabeled MIP-1 β competed -2-fold more effectively for 125 I-MIP- β binding to CCR5.1 than to CC CKR1 (IC_{50} s-100 and 200 nM, respectively).

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At 37°C, 125 I-RANTES bound to both CC CKR1 and CCR5.1 cells at low but significantly increased levels compared to the negative control CC CKR2B cells. Excess unlabeled RANTES reduced 125 I-RANTES binding to CCR5.1 cells (IC_{50} -80nM). The 125 I-RANTES binding sites on CC CKR1 and CCR5.1 could be distinguished by heterologous competition with excess unlabeled MIP-1 α (IC_{50} -20 and 100 nM for CC CKR1 and CCR5.1 respectively).

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Distribution of CCR5 RNA. Compared to other CC CKRs, CCR5 is most like CC CKR2A and CC CKR2B not only in its primary sequence but also in its RNA distribution. Full-length CC CKR2B and CCR5 open reading frame probes recognized a 3.5 kb RNA band by Northern blot hybridization in total RNA made from adherent monocytes. Neither probe recognized RNA in neutrophil or eosinophil samples. To determine whether the CCR5 probe was merely

cross-hybridizing to the monocyte CC CKR2 mRNA, a 30-mer antisense oligonucleotide specific for CCR5 was designed. This probe also detected the 3.5 kb monocyte mRNA species. A similar analysis using specific oligonucleotide probes has indicated that both CC CKR2A and CC CKR2B RNA is present in adherent monocytes.

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Example 3. Cell Fusion Assay Suitable for Drug Screening

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A vaccinia cell fusion system is used to assay the functional ability of CCR5 to confer *env*-mediated fusion competence to CD4-positive nonhuman cells. This assay is carried out as described in Nussbaum *et al.*, 1994, *supra*. In the assay murine NIH 3T3 cells or human HeLa cells are first transfected with the plasmid pSC59.CCR5 and then co-infected with various vaccinia viruses: vTF7-3 (containing the T7 RNA polymerase gene); vCB3 (containing the human CD4 gene); and vaccinia WR (a negative control). A different cell population is co-infected with various vaccinia viruses: vCB-21R (containing the *E. coli lacZ* gene under the transcriptional control of a T7 promoter ($P_{T7}-lacZ$)) along with either a vaccinia virus that encodes a Ba-L *env* gene from a macrophage-tropic isolate or vCB-16 (a negative control, containing a mutant *env* gene encoding an uncleavable, nonfusogenic *unc/env*).

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The cell populations described above are incubated overnight at 31°C to allow expression of the vaccinia-encoded proteins. The cells are washed and mixtures of each combination are prepared in 96-well microtiter plates. Each well contains equal numbers of T7 RNA polymerase-containing cells and *lacZ* gene-containing cells. Replicate plates are incubated for 2-4 hours at 37°C to allow fusion. Samples on one plate are treated with NP-40 and aliquots are assayed for β-galactosidase activity using a 96-well absorbance reader.

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The β-galactosidase assay results from this experiment will show that NIH 3T3 cells coexpressing human CD4 and CCR5 are highly competent for fusion with cells expressing *env* from the macrophage-tropic isolate (Ba-L) but not from a T-cell line-tropic isolate (LAV). In contrast, the data will indicate that NIH 3T3 cells coexpressing human CD4 alone or CCR5 alone are incompetent for fusion with cells expressing *env*. Furthermore, the low background levels of β-galactosidase produced will indicate that NIH 3T3 cells coexpressing human CD4 and CCR5 do not fuse with cells expressing mutant *unc/env*.

In a related experiment, several colonies of stable, transformed mink cells that coexpress human CD4 and CCR5 would be tested for susceptibility to HIV-1 infection by macrophage-tropic or dual-tropic HIV-1 strains (e.g., strains that use CCR5). Transformants containing the human CD4 gene and an irrelevant control gene are used as negative controls. Direct measurements of p24 (HIV core antigen) production will indicate that HIV-1 infection is productive with cells that coexpress human CD4 and CCR5, but not with the negative controls. Moreover, the efficiency of HIV-1 infection of transformed, CD4-positive, CCR5-positive, nonhuman cells is high enough to be detected directly.

Example 4. Anti-CCR5 Antibody Blocks *env*-mediated Membrane Fusion

Based on the known topology of 7-transmembrane segment proteins, four regions of CCR5 are predicted to be exposed at the cell surface. Natural or synthetic peptides are produced or synthesized by methods well-known in the art that correspond to each of these 4 regions. Rabbit antisera is raised by immunization with peptide-KLH (keyhole limpet hemocyanin) conjugates. Total immunoglobulin is purified from the preimmune and the immune sera by chromatographic separation with Protein-A Sepharose. Alternatively, whole cells expressing CCR5 can be used to generate anti-CCR5 antibodies.

Antibodies raised against an 28 amino acid N-terminal portion of CCR5 or against the extracellular loops (e.g., eI-1), can block membrane fusion between macrophage-tropic strains Ba-L, SF162, JR-FL and ADA of HIV and human macrophages, in other words strains that use CCR5. (See, for example, Feng *et al.*, 1996, *supra*; Endres *et al.*, 1996, *supra*).

Example 5. Specificity of CCR5 for *env* From Macrophage-tropic Isolates

The sensitivity of fusion mediated by *env* from different HIV isolates is tested with antibodies prepared against the N-terminal portion of CCR5. The anti-CCR5 antibodies inhibit fusion mediated by the prototypic macrophage-tropic Ba-L *env*, but will not inhibit fusion mediated by the prototypic T-cell line-tropic LAV *env*. The fusion inhibition with anti-CCR5 antibodies is not due to nonspecific inhibitory effects on the cells. Coexpression of CCR5 enhances fusion more with *env* from macrophage-tropic strains (Ba-L, SF162, R-FL, and ADA) than with *env* from T-cell line-tropic isolates (IIIB, LAV, and RF).

Example 6. CCR5 Peptides Block env-mediated Membrane Fusion

Synthetic peptides that correspond to the predicted extracellular loops of CCR5 were prepared and tested for inhibition of env-mediated membrane fusion. Peptides were as follows:

5 extracellular loop-1 (SEQ ID NO:4): LAAQWDFGNTMC

extracellular loop-2 (SEQ ID NO:5): RSQKEGLHYTCSSHFPYSQYQFWK

extracellular loop-3 (SEQ ID NO:6): QEFFFGLNNCSSSNRLD

The peptides were tested using vaccinia-based expression and reporter gene assay system (see Example 3 above). Cell fusion was quantitated by determining the level of β -galactosidase in detergent cell lysates.

10 FIGURE 2 shows that each peptide (0-50 μ g/ml) was able to inhibit fusion between cells expressing the HIV-1 Env from the macrophage-tropic Ba-L isolate and murine cells co-expressing CD4 and CCR5.

Example 7. Cell Lines Expressing CCR5

15 Human HeLa, human embryonic kidney (HEK) 293, and murine NIH 3T3 cell lines (American Type Culture Collection, Rockville, MD) were cultured in DMEM-10 (Dulbecco's modified Eagle's medium [Quality Biologicals, Gaithersburg, MD] containing 10% fetal bovine serum [FBS, HyClone, Logan, UT], 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin). The human PM1 T cell line (Lusso *et al.*, 1995) was obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) and was grown in RPMI-10 (RPMI 1640 medium [Quality Biologicals] containing 10% FBS, 10 mM HEPES, 2mM glutamine, and antibiotics). Recombinant vaccinia virus stocks were prepared by standard procedures (Earl *et al.*, 1991). Pertussis toxin was obtained from List (Campbell, CA). Recombinant chemokines were purchased from Peprotech (Rocky Hill, NJ). Fura 2-AM and propidium iodide were obtained from Molecular Probes (Eugene, OR). Sodium azide and ATP were from Sigma (St. Louis, MO).

CCR5 Constructs. Epitope-tagged variants of CCR5 were created to enable detection by the M5 monoclonal antibody (Kodak, Rochester, NY). The CCR5 open reading frame was amplified by PCR using the following primers: 1) for full-length CCR5 (designated CCR5): a 3'-oligonucleotide containing (from 3' to 5') 27 bases complementary to the last 9 codons of CCR5, 3 bases for the stop codon, 6 bases for an *Xho* I restriction site and 8 miscellaneous bases; 2) for CCR5 lacking most of the cytoplasmic C-terminus (designated CCR₅₀₆): a 3'-oligonucleotide containing (from 3' to 5') 27 bases complementary to codons 298-306 of CCR5, 3 bases for a stop codon, 6 bases for an *Xho* I restriction site and 8 miscellaneous bases; and 3) for both constructs: a 5'-oligonucleotide containing (from 5' to 3') 8 miscellaneous bases, 6 bases for a *Hind* III site, 3 bases for the start codon, 24 bases encoding the flag epitope DYKDDDDK and 27 bases complementary to CCR5 codons 2 to 10. The resulting two PCR products were digested and subcloned between the *Hind* III and *Xho* I sites of the changes using a MSIII fluorimeter (Photon Technology International, S. Brunswick, NJ) in HEK 293 cell lines expressing receptor constructs as previously described. Fuerst, T.R., Niles, E.G., Studier, F.W., and Moss, B. (1986). Briefly, cells were loaded with 2 μ M FURA-2AM at 37°C for 45 min, washed twice and resuspended at 10⁶ cells/ml in HBSS, pH 7.4. Two ml of the cell suspension were placed in a stirred, water-jacketed cuvette at 37°C and excited sequentially at 340 and 380 nm. Fluorescence emission was monitored at 510 nm before and after addition of agonists. For some experiments, cells were incubated with 250 ng/ml pertussis toxin for 3 h prior to functional assay.

Cell Fusion Assay. Fusion between effector cells expressing HIV-1 Env and target cells expressing CD4 was quantitated by a vaccinia-based reporter gene assay in which β -galactosidase is produced selectively in fused cells (Nussbaum *et al.*, 1994). As effector cells, HeLa cells were coinfectected with vCB-21R, which encodes the *E. Coli LacZ* gene under control of the bacteriophage T7 promoter (*J. Virol.* 70, 5487-5494.), and a recombinant vaccinia virus encoding one of the following HIV-1 Envs (*PNAS* 92, 9004-9008.): M-tropic Envs Ba-L (vCB-43; note this is a correction of the nomenclature used for this virus in Broder and Berger, 1995), ADA (vCB-39), SF-162 (vCB-32), and JR-FL (vCB-28); and Unc, an uncleavable mutant of IIIB (vCB-16). In one protocol, the target cells were HEK 293 cell

transfectants stably expressing the indicated CCR5 constructs. These cells were coinfectected with vTF7-3 encoding T7 RN polymerase (Fuerst *et al.*, 1993) and vCB-3 encoding human CD4 (*Cell* 85, 1149-12158.). In both viruses the foreign genes are linked to vaccinia early/late promoters; the multiplicity of infection was 10 pfu/cell for each virus. In another protocol, the target cells were NIH 3T3 cells transfected with pcDNA3-based plasmids encoding CCR5 or CCR5₃₀₆ using DOTAP lipofection (Boehringer Mannheim, Indianapolis, IN); control cells were transfected with pcDNA3 vector alone. After 4 h incubation in DOTAP at 37°C, cells were coinfectected with vTF7-3 and vCB-3; expression of the CCR5 constructs was driven by the T7 promoter. Cell cultures were incubated at 31°C overnight.

Cell surface expression of CCR5 and CCR5₃₀₆ was analyzed by flow cytometry using as the probe either a rabbit polyclonal antiserum generated against a synthetic peptide representing the predicted extracellular amino terminal domain of CCR5 (amino acids 1-28), or a mAb recognizing the Flag epitope. Specific cell surface staining at comparable intensity was obtained when HEK293 cells stably transfected with either CCR5 or CCR5₃₀₆ were incubated with the anti-CCR5 antiserum. In contrast, cells stably transfected with the closely related receptor CCR2b (75% amino acid identity) gave only background fluorescence equivalent to that observed with the signaling is required for the HIV-1 coreceptor activity of CCR5, using a quantitative vaccinia-based reporter gene assay of HIV-1 Env-mediated cell fusion HEK 293 cell transfectants expressing CCR5 or CCR5₃₀₆ (along with vaccinia-encoded CD4) were tested for their ability to fuse with HeLa cells expressing vaccinia-encoded Envs from several M-tropic strains. Comparable levels of fusion occurred with CCR5 and CCR5₃₀₆ for each Env tested. Similar results were obtained in an alternative protocol whereby CCR5 and CCR5₃₀₆ were expressed on NIH 3T3 cells using a transient vaccinia expression system. Thus, the C-terminal truncation that abolished the G protein signal transduction activity of CCR5 had no effect on fusion coreceptor activity.

Pertussis toxin provided an alternative means to test the requirement for G protein signal transduction in the fusion coreceptor activity of CCR5. In the cell fusion assay using the M-tropic Ba-L Env, high concentration of the toxin (500 ng/ml) had no effect on the fusion coreceptor activity of CCR5 expressed transiently in NIH 3T3 cells.

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The effects of pertussis toxin were also tested on productive HIV-1 infection, using the Jurkat-derived T cell line PM1 as the target. PM1 cells express CD4 and are highly susceptible to M-tropic HIV-1 strains. Moreover, CCR5 mRNA is expressed in these cells and infection by M-tropic HIV-1 on PM1 cells is CCR5. In the continuous presence of pertussis toxin (500 ng/ml), robust infection by the M-tropic Ba-L isolate in PM1 cells was observed. Consistent with this, when PM1 cells were used as target cells in the cell fusion assay with effector HeLa cells expressing the Ba-L Env, high levels of fusion activity were observed and this was completely resistant to pertussis toxin. Thus, pertussis toxin at concentrations that potently block G protein-mediated signal transduction had minimal effect on either Env-mediated cell fusion or productive infection. These results parallel earlier reports that pertussis toxin did not process known as receptor sequestration or downmodulation. This process is thought to explain in part the phenomenon of receptor desensitization and could be important either for HIV-1 Env-dependent membrane fusion, and/or chemokine inhibition of fusion. CCR5 was strongly downmodulated by chemokine ligands, whereas the truncated CCR5₃₀₆ receptor was unaffected. This indicates that, in addition to containing critical determinants of signaling, the C-terminal domain of CCR5 also contains critical determinants for chemokine-mediated down-modulation.

It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

What Is Claimed Is:

1. A recombinant cell line that expresses CCR5 polypeptide.
2. The cell line of claim 1, wherein the cells coexpress human CD4 and CCR5.
3. A recombinant host cell stably transformed with a polynucleotide encoding CCR5 polypeptide, wherein the cell co-expresses CCR5 and CD4 polypeptide.
4. A recombinant host cell stably transformed with a polynucleotide encoding CCR5 polypeptide and a polynucleotide encoding CD4 polypeptide, wherein the cell co-expresses CCR5 and CD4 polypeptide.
5. The cell as in any of claims 1-4, wherein the cell is a human cell.
6. The cell as in any of claims 1-4, wherein the cell is a non-human cell.
7. An antibody which specifically binds to CCR5 polypeptide or fragments thereof.
8. The antibody of claim 7, wherein the antibody blocks membrane fusion between HIV and a target cell.
9. The antibody of claim 7, wherein the antibody is a monoclonal antibody.
10. The antibody of claim 7, wherein the antibody blocks chemokine binding.
11. A cell that expresses a CCR5 gene, wherein the CCR5 gene is not stably integrated into the genome of the cell.
12. The cell of claim 11, wherein the cell also expresses a CD4 gene.

13. An isolated polynucleotide which encodes an amino acid sequence as set forth in SEQ ID NO:2.
14. An isolated polynucleotide which encodes an amino acid sequence as set forth in SEQ ID NO:4.
15. An isolated polynucleotide selected from the group consisting of:
 - a) SEQ ID NO:1;
 - b) SEQ ID NO:3;
 - c) SEQ ID NO:1, wherein T can also be U;
 - d) SEQ ID NO:3, wherein T can also be U;
 - e) nucleic sequences complementary to SEQ ID NO:1;
 - f) nucleic sequences complementary to SEQ ID NO:3;
 - g) fragments of a), c), or e) that are at least 15 bases in length and that will selectively hybridize to genomic DNA which encodes the CCR5 protein of SEQ ID NO:2; and
 - h) fragments of b), d), or f) that are at least 15 bases in length and that will selectively hybridize to genomic DNA which encodes the CCR5 protein of SEQ ID NO:4.
16. An expression vector containing in operable linkage the polynucleotide as in claim 14.
17. A host cell containing the vector of claim 16.
18. A substantially purified peptide fragment of CCR5, wherein the peptide inhibits cell membrane fusion between HIV and a target cell or between an HIV-infected cell and a CD4 positive uninfected cell.
19. The peptide of claim 18, wherein the peptide is the amino acid sequence as set forth in SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and conservative variations thereof.

20. A substantially purified CCR5-binding agent, wherein the binding agent inhibits membrane fusion between HIV and a target cell or between an HIV-infected cell and a CD4 positive uninfected cell.
21. The agent of claim 20, wherein the agent is selected from a biologic agent and a chemical compound.
22. The agent of claim 20, wherein the biologic agent is a chemokine.
23. The agent as in claims 18 or 20, wherein the agent blocks chemokine binding to CCR5.
24. A method of inhibiting membrane fusion between HIV and a target cell or between an HIV-infected cell and a CD4 positive uninfected cell comprising contacting the target or CD4 positive cell with a fusion-inhibiting effective amount of a CCR5 binding or blocking agent.
25. The method of claim 24, wherein the agent is a anti-CCR5 antibody or epitope binding fragment thereof.
26. The method of claim 25, wherein the antibody is a monoclonal antibody or a polyclonal antibody.
27. The method of claim 24, wherein the contacting is by *in vivo* administration to a subject.
28. The method of claim 25, wherein the anti-CCR5 antibody is administered by intravenous, intra-muscular or subcutaneous injections.

29. The method of claim 27, wherein the anti-CCR5 antibody is administered within a dose range of 0.1 ug/kg to 100 mg/kg.
30. The method of claim 28, wherein the antibody is formulated in a pharmaceutically acceptable carrier.
31. A method for identifying a compound which binds to CCR5 polypeptide comprising:
 - a) incubating components comprising the compound and CCR5 polypeptide under conditions sufficient to allow the components to interact; and
 - b) measuring the binding of the compound to CCR5 polypeptide.
32. The method of claim 31, wherein the compound is a peptide.
33. The method of claim 31, wherein the compound is a peptidomimetic.
34. The method of claim 31, wherein the CCR5 polypeptide is expressed in a cell.
35. The method of claim 34, wherein the cell is the cell of claim 1.

36. A method for identifying a compound which blocks membrane fusion between HIV and a target cell or between an HIV-infected cell and a CCR5 positive uninfected cell comprising:
 - a) incubating components comprising the compound and a CCR5 positive cell under conditions sufficient to allow the components to interact;
 - b) contacting the components of step a) with HIV or an HIV-infected cell; and
 - c) measuring the ability of the compound to block membrane fusion between HIV and the CCR5 positive cell or between an HIV-infected cell and a CCR5 positive uninfected cell.
37. The method of claim 36, wherein the CCR5 positive cell is a CD4 positive cell.
38. The method of claim 36, wherein measuring the ability of the composition to block membrane fusion is by detection of a reporter means.
39. The method of claim 38, wherein the reporter means is selected from the group consisting of a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator, or an enzyme.
40. The method of claim 39, wherein the reporter means is a *lacZ* gene.
41. A transgenic non-human animal having a phenotype characterized by expression of CCR5 polypeptide and CD4 polypeptide otherwise not naturally occurring in the animal, the phenotype being conferred by a transgene contained in the somatic and germ cells of the animal, the transgene comprising a nucleic acid sequence which encodes CCR5 polypeptide and a nucleic acid sequence which encodes CD4 polypeptide.

42. The transgenic non-human animal of claim 41, wherein the animal is a mouse.
43. The transgenic non-human animal of claim 41, wherein the animal is a rabbit.
44. A transgenic non-human animal having a phenotype characterized by expression of CCR5 polypeptide otherwise not naturally occurring in the animal, the phenotype being conferred by a transgene contained in the somatic and germ cells of the animal, the transgene comprising a nucleic acid sequence which encodes CCR5 polypeptide.
45. A method for producing a transgenic non-human animal having a phenotype characterized by expression of CCR5 polypeptide and CD4 polypeptide otherwise not naturally occurring in the animal, the method comprising:
 - (a) introducing at least one transgene into a zygote of an animal, the transgene(s) comprising a DNA construct encoding CCR5,
 - (b) transplanting the zygote into a pseudopregnant animal,
 - (c) allowing the zygote to develop to term, and
 - (d) identifying at least one transgenic offspring containing the transgene.
46. The method of claim 45, further comprising a DNA construct encoding CD4.
47. The method of claim 45, wherein the introducing of the transgene into the embryo is by introducing an embryonic stem cell containing the transgene into the embryo.
48. The method of claim 45, wherein the introducing of the transgene into the embryo is by infecting the embryo with a retrovirus containing the transgene.
49. The method of claim 45, wherein the animal is selected from the group consisting of a mouse and a rabbit.

50. A transgenic non-human animal having a transgene disrupting or interfering with expression of CCR5 chromosomally integrated into the germ cells of the animal.
51. The transgenic animal of claim 50, wherein the animal is selected from the group consisting of a mouse and a rabbit.
52. The transgenic non-human animal of claim 50, wherein the transgene comprises CCR5 antisense polynucleotide.
53. A method of treating a subject having or at risk of having an HIV infection or disorder, comprising administering to the subject, a therapeutically effective amount of an anti-CCR5 antibody, wherein the antibody inhibits cell-cell fusion in cells infected with HIV.
54. The method of claim 53, wherein the antibody is a monoclonal antibody.
55. The method of claim 54, wherein the monoclonal antibody is a humanized monoclonal antibody.
56. The method of claim 53, wherein the monoclonal antibody is administered to a patient suffering from AIDS or ARC.
57. The method of claim 53, wherein the monoclonal antibody is administered within a dose range between about 0.1/kg to about 100 mg/kg.
58. The method of claim 53, wherein the monoclonal antibody is formulated in a pharmaceutically acceptable carrier.

59. A method of treating a subject having an HIV-related disorder associated with expression of CCR5 comprising administering to an HIV infected or susceptible cell of the subject, a agent that suppresses CCR5.
60. The method of claim 59, wherein the agent is an anti-CCR5 antibody.
61. The method of claim 59, wherein the agent is an antisense nucleic acid that hybridizes to a CCR5 nucleic acid.
62. The method of claim 59, wherein the agent is introduced into the cell using a carrier.
63. The method of claim 59, wherein the carrier is a vector.
64. The method of claim 59, wherein the administering is *ex vivo*.
65. The method of claim 59, wherein the administering is *in vivo*.
66. A pharmaceutical composition comprising CCR5 in a pharmaceutically acceptable carrier.
67. A method for inhibiting the expression of CCR5 in a cell comprising contacting CCR5 with an inhibiting effective amount of an antisense oligonucleotide that binds to a segment of an mRNA transcribed from a CCR5 gene, whereby the binding of the antisense to the mRNA segment inhibits CCR5 expression.
68. Substantially pure CCR5 polypeptide having an amino acid sequence as set forth in SEQ ID NO:4.

19

214

SEQ TO NO: 1

1 31 11
 CTC GTC CGC AGG CCC ATT ATG CAT CAA TGG CAA GAA ACT CTC GTC GCG GGC TGG AAC AAC GAC ATC
 61 / 21 91 31
 GAT TAT CAA GGC TCA ACT CCA ATG TAT GAC ATC AAT TAT TAT ACA TGG GAS CCC TGG CAA
 121 / 61 191 / 51
 GSD TGT CIN Val Ser Ser Pro Ile Tyr Ser 110 ASN Tyr Tyr Thr Ser Glu Ser Cys Glu
 181 / 61 211 / 71
 ATC TTT GOT TTT GTG GGC AAC ATT CTC GTC CTC ATG CTC ATA AAC TCC AAA AGG CTC
 241 / 61 271 / 91
 Ile Phe Gly Phe Val Gly Ser Met Leu Val 110 Ile 110 Ile 110 Ser Cys Iys Arg Leu
 301 / 161 331 / 111
 AAC AGC ATG ACT GAC ATT TAC CTC CTC AAC CTC GCT ATC TCT GAC CTC TCT TTC CTT CTT
 Lys Ser Met Thr Asp 110 Tyr Ile Ile Ser Leu Glu 110 Ser Asp Leu Phe Phe Ile Ile
 421 / 161 451 / 151
 ACT CTC CCC TTT TCC CCT CAC TAC TGG CTC GTC GAC TGG CAA ATT ACA ATC ATC TCT
 Thr Val Pro Phe Trp Ala Histidine Ala Glu Trp Asp Phe Gly Ser Thr Met Cys
 481 / 181 511 / 171
 CAA CTC TGG ACA GAG CTC TAT TTT ATA GGC TTC TCC TCT GGA ATC CTC CTC ATC ATC CTC
 Glu Leu Leu Thr Gly Ile Tyr Phe Ile Gly Phe Ser Gly Ile Phe Phe Ile Ile Ile Ile
 561 / 181 591 / 171
 CTC ACA ATC CAT ACC TAC CTC GCG GTC CTC CAT GOT TTT CCT TTA AAA GGC AGG AGG
 Ile CTC 110 Asp Arg Tyr Leu Ala Val Val His Asp Val Phe Ala Ile Ile Ile Ile Arg Thr
 641 / 181 671 / 171
 GTC AAC TTT GCG GTC CTC ACA ACT CTC ATC ACT TGG CTC CTC CCT CTC TTT CGG TTT CTC
 Val Thr Phe Gly Val Val Thr Ser Val 110 Thr Trp Val Val Ala Val Phe Ala Ser Ile
 721 / 181 751 / 191
 CCA GCA ATC ATC TTT ACC AGA TCT CAA AAA GAA CCT CTC CAT TAC ACC TAC ADC TCT CAT
 Pro Gly 110 Ile Thr Thr Arg Ser Glu Iys Glu Ile His Tyr Thr Cys Ser Ser His
 801 / 201 831 / 221
 TTT CCA TAC AGT AAC TAT CAA TTC TGG AAC ATT TTC CAG ACA TTA AAC ATA GTC ATC TTC
 Phe Pro Tyr Ser Glu Tyr Glu Phe Trp Iys Ser Phe Glu Thr Ile Ile Val 110 Leu
 861 / 221 891 / 221
 GGG CTC CTC CGG CTC CTC ATT TGG ATC CTC ATC TCC TAC TGG CCA ATC CCA AAA ATT CTC
 Gly Leu Val Ile Uro Leu Leu Val Met Val 110 Cys Tyr Ser Gly Ile Ile Ile Iys Thr Ile
 931 / 241 951 / 251
 CTC CGG TGT CCA ATT GAG AAC AAC AGG CAC AGG GCG UTRG AGG CTC ATC TTC AAC ATC ATC
 101 Arg Cys Arg Ser Glu Lys Iys Arg His Arg Ala Arg Ile Ile Phe Thr Ile Met
 1081 / 361 1111 / 271
 ATT CTT TAT TTT CTC TGG TCC CCT CCT TAC AAC ATT GTC CCT CTC CTC AAC AAC TTC CAG
 116 Val Tyr Phe Leu Phe Trp Ala Pro Tyr Asn 110 Val Ile Ile Ile Ile Ile Ile Ile Ile
 1241 / 381 1271 / 291
 GAA TTC TTT GGC CTC ATT ATT ATT TCC AGT AGC TCT AAC AGG TTC GAC CAA GCT ATC CAG GTC
 Glu Phe Phe Gly Leu Asn Ser Cys Ser Ser Ser Ser Arg Ile Asp Glu Asp Met Glu Val
 1301 / 301 1331 / 311
 ACA CAC ACT CTT TGC ATC ACC CAC TCC TGC ATC AAC CCC ATC ATC ATT CCT TTT GTC CGG
 Thr Glu Ile Glu Phe Met Thr His Cys Cys Ile Asn Pro Ile 110 Tyr Ala Phe Val Gly
 1461 / 331 1491 / 331
 GAG AAC TTC AGA AAC TAC CTC TTA GTC TTC TTC CAA AAC AAC CTC ATT CCT AAA CGG TTC TCC
 Glu Iys Phe Arg Ser Tyr Ile Ile Val Phe Phe Glu Iys His Ile Ile Ile Iys Arg Phe Cys
 1523 / 343 1553 / 331
 AAA TOC TGT TCT ATT TTC CAG URA GAG CCT CCT GAG CCA AGC TCA GTC ATT TAC ACC CCA
 Lys Cys Cys Ser Ile Phe Glu Cys Glu Ala Pro Glu Arg Ala Ser Ser Val Tyr Thr Ser
 1681 / 381 1711 / 371
 TCC ACT GGG GAG CGG GAA ATA TTT GTC CGG TTD ATG AAC TAC CCA CTC AAC TGG CCT CCT GAC
 Ser Ser Cys Glu Glu Glu Ile Ser Val Glu Ile
 1741 / 383 1771 / 391
 CCA GTC AGA GTC GTC CAC ATT GCT TAC CTC TCA TAC ACA CCC TGG CCT GTC CGG GTC
 Pro Val Arg Val Val His Met Ala AMB Phe Ser Tyr Thr Ala Trp Ala Gly Gly Ile Val
 1821 / 401 1851 / 411
 GCA GAG CTC TTT TTT AAA AGG AAC AGG TTA CTC TTA TAG AGC GTC TAA CAT TCA TCC ATC
 Gly Glu Val Phe Phe Iys Arg Iys Ile Ile

Figure 1F

Figure 1C

SEQ ID NO:3

I aagaacacici ccccggggggg aacaagaagg aataicaagi gicaagicca aiciaigaca
61 icaaattaa iacaicggag cccicccaaa azaicaalgi gaagcaalc gcagccccgc
121 iccicccccc gctictacica cggggpcia icttgggti tgiggggcaac aigctggica
181 icccicccci galaaacigc aaaaggciga agaecaigac igacaaciac cggcicaacc
241 tggccalcic lgaccipm nccctttia cggccccci cggggcac lacgctgccc
301 cccagggga ctggggaa acaaatggic aacitctngac agggeciciat ittaaggcc
361 icccicgg aatcccttc aicaacccic lgacaacigc tagtgaccig cggcggcc
421 aigctgigt ipctttaaaa gcccaggacgg icaccctgg ggiggigaca agigtpaica
481 ctggggggp gctcigpm gctcicccicc caggaatcat ctttaccaga icicaaaaap
541 aaggcicca itaacccigc aigcicicat nccalaacag icagtaicca ncttggaaaga
601 atttccagac aataaagata gicaicctgg gctcggccctt gcccggcpi gicaiggtca
661 icicciacigc ggaaatccia aaaaacccigc ncttggggicg aaatggagaag aagaggcaca
721 gggcigigag gctttaiccc accaialcaigc ncttattt icicicgg gctccctaca
781 acatggccci icicciagaac accnccagg aatcttgg ccigaataal tgcagtlageci
841 ctaacaggpi ggaccaagci aigcaggiga cagagacici tggttttgacg cactggcga
901 tcaaccccait caiciaigcc mpcggggg aagaatggc aacaiaccic taatggcc
961 tccaaaagca catggccaaa cgccicgca aigcigicca tattttccag caagaggcc
1021 ccgagcggc aagcicagti iacaccggat ccacggggga gcaggaaata tcttggggcc
1081 tggpacacgg acicaaggg gctgggggacc cagtcagatg tggttttttgcagttt
1141 catacacacgc ctggggcgggg ggiggggggg gagaggcici ttttttttttttttt
1201 talagagggti ctaagatca icca

SEQ ID NO:4

MDYQVSSPIYDINYTSEPCQKINVQIAARLLPPLYSLVIFGFVGNMLVILILINCKRL
KSMTDIYLLNLAISDLFFLLTVPFWAHYAAAQWDFGNTMCQ
LLTGlyFIGFFSGIFFIILLTIDRYLAVVHAVFALKARTVTFGVVTSVITWWVAVFAS
LPGIIFTRSQKEGLHYTCSSHFPYSQYQFWKNFOTLKIVILGLVLPLLMVICYSGIL
KTLLRCRNEKKRHR AVR LIFTIMIVYFLFWAPYNIVLLLNTFQEFGLNNCSSNRD
QAMQVTETLGMTHCCINPIIYAFVGEEKFRNYLLVFFOKHIAKRFCKCCSIFQQEAPER
ASSVYTRSTGEQEISVGL

4/4

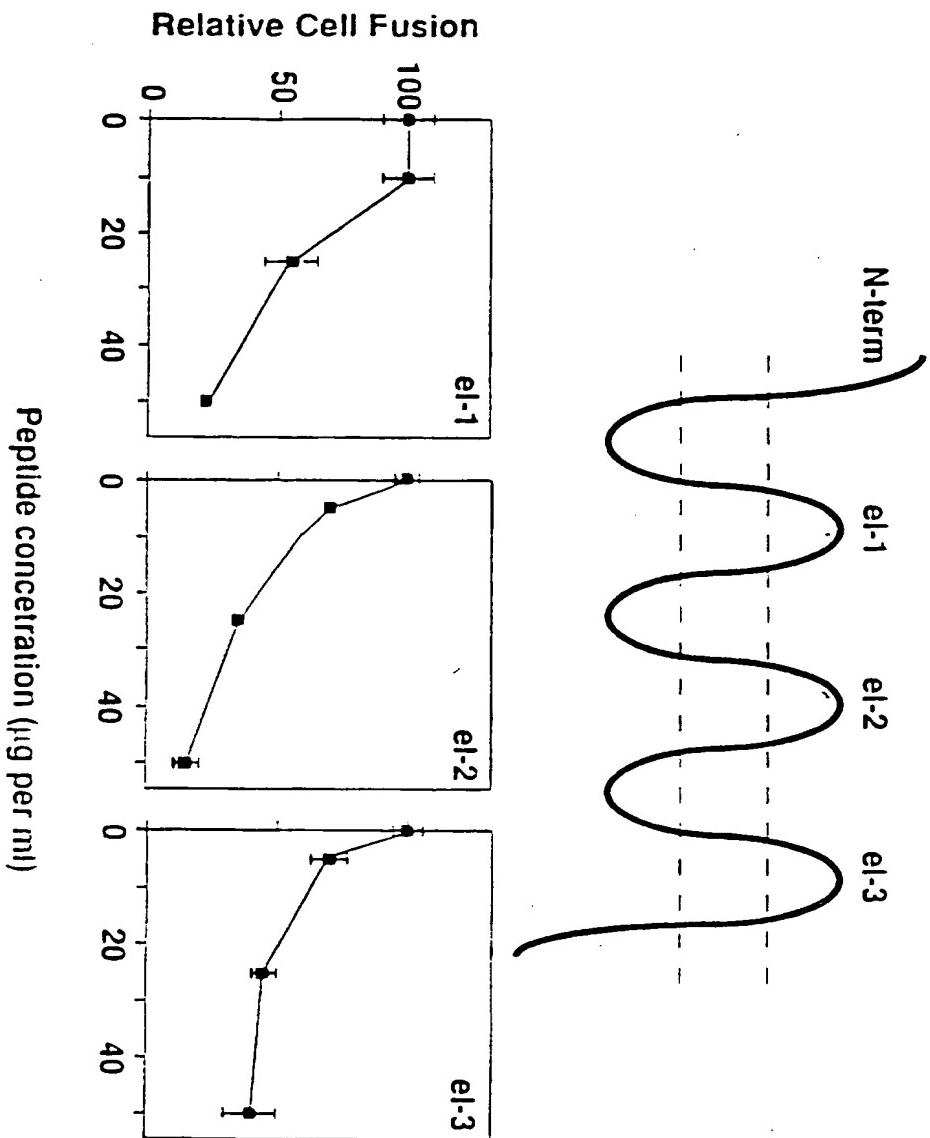


FIGURE 2



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :		A3	(11) International Publication Number: WO 97/45543
C12N 15/12, A61K 38/17, C07K 14/715, G01N 33/68, C07K 16/28, A01K 67/027			(43) International Publication Date: 4 December 1997 (04.12.97)
(21) International Application Number: PCT/US97/09586		ALKHATIB, Ghalib [-/US]; Bethesda, MD (US). MURPHY, Philip, M. [-/US]; Rockville, MD (US). BRODER, Christopher, C. [-/US]; Rockville, MD (US). KENNEDY, Paul, E. [-/US]; 8502 Flower Avenue, Takoma Park, MD 20912 (US).	
(22) International Filing Date: 28 May 1997 (28.05.97)		(74) Agent: HAILE, Lisa, A.; Fish & Richardson, P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).	
(30) Priority Data: 60/018,508 28 May 1996 (28.05.96) US		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Application or Grant (63) Related by Continuation US Filed on 60/018,508 (CIP) 28 May 1996 (28.05.96)		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (<i>for all designated States except US</i>): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF HEALTH AND HUMAN SERVICES, NATIONAL INSTITUTES OF HEALTH [US/US]; Office of Technology Center, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).		(88) Date of publication of the international search report: 7 May 1998 (07.05.98)	

(54) Title: CC CHEMOKINE RECEPTOR 5, ANTIBODIES THERETO, TRANSGENIC ANIMALS

(57) Abstract

The susceptibility of human macrophages to human immunodeficiency virus (HIV) infection depends on cell surface expression of the human CD4 molecule and CC cytokine receptor 5. CCR5 is a member of the 7-transmembrane segment superfamily of G-protein-coupled cell surface molecules. CCR5 plays an essential role in the membrane fusion step of infection by some HIV isolates. The establishment of stable, nonhuman cell lines and transgenic mammals having cells that coexpress human CD4 and CCR5 provides valuable tools for the continuing research of HIV infection. In addition, antibodies which bind to CCR5, CCR5 variants, and CCR5-binding agents, capable of blocking membrane fusion between HIV and target cells represent potential anti-HIV therapeutics for macrophage-tropic strains of HIV.

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INTERNATIONAL SEARCH REPORT

Internat Application No

PCT/US 97/09586

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/715 C07K16/28 A61K38/17 G01N33/68
A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAMSON, M. ET AL.: "Molecular cloning and functional expression of a new human CC-chemokine receptor gene" BIOCHEMISTRY, vol. 35, no. 11, 19 March 1996, pages 3362-3367, XP002055726 cited in the application see the whole document	1,5,6, 11, 14-17, 20-22, 31-35, 66,68
Y		2-4, 7-10,12, 23-30, 36-49, 53-60, 62,64,65
	---	-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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3

Date of the actual completion of the international search

20 February 1998

Date of mailing of the international search report

17.03.98

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INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/US 97/09586

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FENG Y ET AL: "HIV-1 ENTRY COFACTOR: FUNCTIONAL CDNA CLONING OF A SEVEN-TRANSMEMBRANE, G PROTEIN-COUPLED RECEPTOR" SCIENCE, vol. 272, 10 May 1996, pages 872-877, XP002027999 cited in the application see page 875; figure 6 see page 876, left-hand column, paragraph 2 - middle column	2-4, 7-10,12, 23-30, 36-49, 53-60, 62,64,65
X	COMBADIERE, C. ET AL.: "Cloning and functional expression of a human eosinophil CC chemokine receptor." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, 1995, page 30235 XP002055727 cited in the application see the whole document & COMBADIERE, C. ET AL.: "Cloning and functional expression of a human eosinophil CC chemokine receptor." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, 1995, pages 16491-16494, XP002037624 cited in the application see the whole document	1,5,6, 11, 14-17, 20-22, 31-35
X	DATABASE PIR2 Entry/accession number G02653, 21 December 1990 COMBADIERE, C.: "CC chemokine receptor 5 - human" XP002056291 see the whole document	1,5-7,9, 11,13, 15-17,66
X	COCHI F ET AL: "IDENTIFICATION OF RANTES, MIP-1ALPHA, AND MIP-1BETA AS THE MAJOR HIV-SUPPRESSIVE FACTORS PRODUCED BY CD8+ T CELLS" SCIENCE, vol. 270, 15 December 1995, pages 1811-1815, XP000616644 cited in the application see the whole document	20-24, 27,36-38
A		1-17, 28-35, 39-68
X	EP 0 310 136 A (UNIV ROCKEFELLER) 5 April 1989 see page 26; claim 64	23
		-/-

INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/US 97/09586

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GONG J -H ET AL: "RANTES AND MCP-3 ANTAGONISTS BIND MULTIPLE CHEMOKINE RECEPTORS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 18, 3 May 1996, pages 10521-10527, XP002047804 see the whole document ---	20-23
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P,X	RUCKER, J. ET AL.: "Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity." CELL, vol. 87, 1 November 1996, pages 437-446, XP002055730 see page 441, left-hand column, paragraph 2 see page 443, left-hand column, paragraph 2 - page 444, right-hand column, paragraph 1 ---	1-6,11, 12,14-19
P,X	WO 96 39437 A (HUMAN GENOME SCIENCES INC ; LI YI (US); RUBEN STEVEN M (US)) 12 December 1996 see the whole document ---	1,5-11, 13-18, 31-35, 53,54, 56-60, 66,68
P,X	COMBADIERE, C. ET AL.: "Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1alpha, MIP-1beta, and RANTES." JOURNAL OF LEUKOCYTE BIOLOGY, vol. 60, July 1996, pages 147-152, XP002055731 see page 149; figure 1 see page 147, line R, paragraph 2 ---	1,5,6, 11,13, 15-17, 20-22, 31-35,66
E	WO 97 22698 A (ICOS CORP) 26 June 1997 see the whole document ---	1-12, 14-40, 53,54, 56-68

-/-

INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/US 97/09586

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 97 32019 A (EUROSCREEN S A ;SAMSON MICHEL (FR); PARMENTIER MARC (BE); VASSART) 4 September 1997 see page 32, line 14 - line 18 see page 44 - page 59 see page 10, line 4 - page 17, line 22 see page 7, line 6 - page 8, line 28 see page 4, line 2 - line 19 ---	1-12, 14-18, 20-39, 41-44, 50-54, 56-68
E	WO 97 37005 A (PROGENICS PHARM INC) 9 October 1997 see page 30, paragraph 3 - page 36 see page 47 - page 53 ---	1-6,11, 12, 14-17, 20-24, 36-39,59
E	WO 97 44055 A (UNIV NEW YORK) 27 November 1997 see the whole document -----	1-9,11, 12, 14-17, 20-22, 24-39, 41-44, 53-61, 64-68

INTERNATIONAL SEARCH REPORT

In. International application No.
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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/09586

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 24-30, 53-65 and 67 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.